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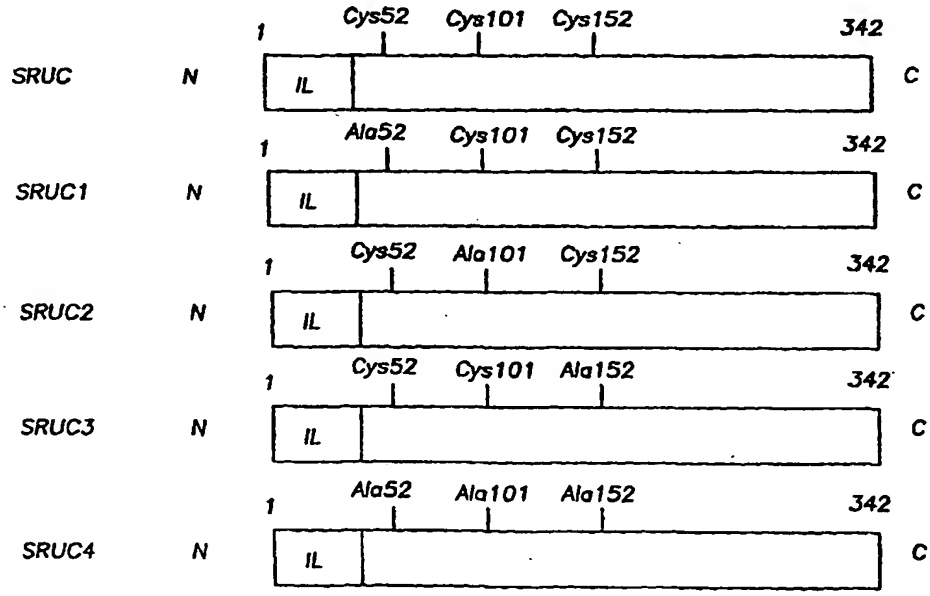


(57) Abstract

A polynucleotide encoding a secreted form of wild type *Renilla* luciferase. Also provided is a polynucleotide encoding a secreted modified form of wild type *Renilla* luciferase. Additionally, the polypeptides encoded by the polynucleotides of the present invention and uses of the polynucleotides and polypeptides of the present invention in biological assays. Also, a stable mammalian packaging cell line which produces retroviruses carrying a polynucleotide encoding a secreted *Renilla* luciferase.

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# SECRETED *RENILLA* LUCIFERASE

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under cooperative agreement number DAMD17-97-2-6017 with the United States Department of the Army, United States Army Research Acquisition Activity. The Government has certain rights in this invention.

## CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application is a continuation of United States Patent Application 09/330,317 filed June 10, 1999, entitled "Secreted *Renilla* Luciferase"; a continuation-in-part of United States Provisional Patent Application 60/099,214 filed September 4, 1998, entitled "Secreted *Renilla* Luciferase"; and a continuation-in-part of United States Patent Application 09/152,031 filed September 11, 1998, entitled "Artificial Chromosomes, Uses Thereof and Methods for Preparing Artificial Chromosomes"; which is a continuation-in-part of United States Patent Application 08/695,191 filed August 7, 1996, entitled "Artificial Chromosomes, Uses Thereof and Methods for Preparing Artificial Chromosomes"; which is a continuation-in-part of United States Patent Application 08/682,080 filed July 15, 1996, entitled "Artificial Chromosomes, Uses Thereof and Methods for Preparing Artificial Chromosomes"; which is a continuation-in-part of United States Patent Application 08/629,822 filed April 10, 1996, entitled "Artificial Chromosomes, Uses Thereof and Methods for Preparing Artificial Chromosomes," the contents of which are all incorporated by reference herein in their entirety.

## BACKGROUND

Genes encoding proteins with readily detectable activities, referred to as reporter proteins, are routinely used in biological assays to study a variety of biological events. These biological events include gene expression, gene transfer and the intracellular movement of molecules. Genes encoding reporter proteins which are capable of generating light emission such as luciferases, in particular, have been incorporated into sensitive, noninvasive biological assays which are simple to perform. The most widely used luciferases used in assays are encoded by genes from the bioluminescent *Vibrio* bacteria, the jellyfish *Aequoria victoria*, and the firefly *Photinus pyralis*.

Further, genes encoding reporter proteins that are secreted offer distinct advantages when they are incorporated into assays because they permit the monitoring of gene expression over time without destroying the cells or tissues which are being studied. Secreted alkaline phosphatase (SEAP) is an example of a light-emitting, secreted reporter protein whose encoding gene is useful in mammalian cell assays (see, for example, Yang, T.T. et al. "Quantification of gene expression with a secreted alkaline phosphatase reporter system." *Biotechniques*, 23: 1110-1114 (1997)). Other light-emitting, secreted reporter proteins are known, including the luciferase from the marine ostracod *Vargula hilgendorfii*, or from the decapod shrimp *Oplophorus*, but their use is restricted because either the luciferin substrates for their secreted proteins are not commercially available or their encoding genes have not been cloned.

Additionally, dual reporter assay systems are often used for studies of gene expression in transiently transfected cells. In these systems, one reporter gene is fused to a DNA promoter element of interest, while the other reporter gene is fused to a constitutive promoter. Measurements obtained from the expression of the latter are then taken for normalization between experiments. A good dual reporter system should offer assays that are both sensitive and simple to perform.

Therefore, it would be useful to have other genes encoding secreted reporter proteins which could be incorporated into biological assays and which have commercially available substrates for the secreted reporter proteins they encode. Further, it would be useful to have vectors containing these genes to prepare kits for performing the assays. Additionally, it would be useful to have a dual reporter system that is both sensitive and simple to perform.

## SUMMARY

In one embodiment, there is provided a polynucleotide encoding a secreted *Renilla* luciferase. According to another embodiment of the present invention, there is provided a secreted *Renilla* luciferase.

According to still another embodiment of the present invention, there is provided a method of performing a biological assay. The method comprises providing a polynucleotide encoding a secreted *Renilla* luciferase. The method can additionally comprise transfecting a host cell, such as a mammalian cell, with the polynucleotide. The method can also comprise detecting light emission from the *Renilla* luciferase coded by the polynucleotide that has been

secreted in the culture media in which the host cell is growing. The method can further comprise transfecting the host cell with a second polynucleotide encoding a second light emitting protein such as *seap*. The method can also comprise detecting light emission from the second light emitting protein coded by the second polynucleotide that has been secreted in the culture media.

According to another embodiment of the present invention, there is provided a plasmid or a vector containing a polynucleotide according to the present invention, as well as a host cell, such as a mammalian cell, transfected with a polynucleotide according to the present invention. The present invention can also include a kit for performing a biological assay, comprising a polynucleotide according to the present invention. The kit can include a second polynucleotide, such as *seap*.

According to another embodiment of the present invention, there is provided a stable mammalian packaging cell line which produces retroviruses carrying a polynucleotide encoding a secreted *Renilla* luciferase.

## FIGURES

These and other features, aspects and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying figures where:

Figure 1 shows a partial schematic diagram of the plasmid pLNCX-RUC;

Figure 2 shows a partial schematic diagram of the plasmid pLNCX-SRUC;

Figure 3 shows a graph of the stability of isolated *Renilla* luciferase in cell culture media and in whole blood;

Figure 4 shows a bar graph of the *Renilla* luciferase activity in cell lysates for mammalian cells transfected with (1) pLNCX, (2) pLNCX-RUC, or (3) pLNCX-SRUC;

Figure 5 shows a bar graph of the *Renilla* luciferase activity in cell culture media of mammalian cells transfected with (1) pLNCX, (2) pLNCX-RUC, or (3) pLNCX-SRUC;

Figure 6 shows a graph of the time course appearance of *Renilla* luciferase activity in cell culture media of mammalian cells transfected with pLNCX, pLNCX-RUC, or pLNCX-SRUC;

Figure 7 shows an immunoblot analysis for the presence of *Renilla* luciferase in total cell lysates for mammalian cells transfected with plasmid pLNCX, pLNCX-SRUC, or

pLNCX-SRUC;

Figure 8 shows an immunoblot analysis for the presence of *Renilla* luciferase in cell culture media of mammalian cells that were transfected with plasmid pLNCX, pLNCX-SRUC, or pLNCX-SRUC;

Figure 9 shows a schematic diagrams of the amino acid sequences of the secreted *Renilla* luciferase preproteins, SRUC, SRUC1, SRUC2, SRUC3 and SRUC4;

Figure 10 shows an immunoblot analysis for the presence of *Renilla* luciferase in total cell lysates of mammalian cells transfected with expressing genes encoding SRUC (lane 1), SRUC1 (lane 2), SRUC2 (lane 3), SRUC3 (lane 4), and SRUC4 (lane 5), and isolated from *Escherichia coli* expressing the *Renilla* luciferase cDNA as control (lane 6);

Figure 11 shows a bar graph depicting the measured *Renilla* luciferase bioluminescence activity in mammalian cell lysates for cells transfected with SRUC1 (lane 1), SRUC2 (lane 2), SRUC3 (lane3), SRUC4 (lane 4) and SRUC (lane 5);

Figure 12 shows a bar graph depicting the measured *Renilla* luciferase bioluminescence activity in culture media of cells transfected with SRUC1 (lane 1), SRUC2 (lane 2), SRUC3 (lane3), SRUC4 (lane 4) and SRUC (lane 5);

Figure 13 shows a graph of the time course appearance of *Renilla* luciferase activity in cell culture media of mammalian cells transfected with pND2-SRUC, pND2-SRUC1, pND2-SRUC2, pND2-SRUC3, and pND2-SRUC4;

Figure 14 shows a bar graph of the *Renilla* luciferase activity for cell lysates and culture medium of cells grown in DMEM supplemented with 10% FBS and cells grown serum free medium supplemented with 1% FBS;

Figure 15 shows a graph of stability of bioluminescence activity of SRUC and SRUC3 that has already been secreted by mammalian cells;

Figure 16 shows a bar graph of the luciferase activity in culture medium of cells co-transfected with *sruc3* and *seap*;

Figure 17 shows a bar graph of the alkaline phosphatase activity in culture medium of cells co-transfected with *sruc3* and *seap*; and

Figure 18 shows a chart of the range of *Renilla* luciferase activities measured in the culture media of stable packaging cell lines transduced with retroviruses carrying the *sruc3* gene.

## DESCRIPTION

*Renilla reniformis* is an anthozoan coelenterate living in shallow coastal waters of North America which displays blue-green bioluminescence. This bioluminescence results from a monomeric luciferase enzyme ($M_r$ 36,000) which catalyzes the oxidative decarboxylation of the complex organic molecule coelenterazine (the luciferin) in the presence of dissolved oxygen to yield oxyluciferin, $CO_2$, and blue light ($\lambda_{max}$=480 nm) in vitro. In vivo, the *R. reniformis* energy transfer occurs from the luciferase-bound oxyluciferin excited state donor to a green fluorescent protein acceptor resulting in blue-green light emission ($\lambda_{max}$=509 nm). A polynucleotide sequence, SEQ ID NO:1, encoding a functional full-length *Renilla* luciferase protein SEQ ID NO:2 has been cloned (Lorenz et al., "Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase." *Proc. Natl. Acad. Sci. USA*, May 15; 88(10):4438-4442 (1991)).

According to one embodiment of the present invention, there is provided a polynucleotide sequence encoding a naturally occurring light-emitting reporter protein which has an amino acid sequence added so that it is secreted by mammalian cells. According to another embodiment of the present invention, there is provided a polynucleotide sequence encoding a modified form of a naturally occurring light-emitting reporter protein which additionally has an amino acid sequence added so that it is secreted by mammalian cells. The invention also includes the secreted light-emitting reporter proteins which are encoded by the polynucleotides of the present invention. The invention further includes methods of using the polynucleotides and polypeptides of the present invention, and kits containing the polynucleotides of the present invention which can be used to perform biological assays of biological events such as gene expression, gene transfer and the intracellular movement of molecules within multicellular organisms, among other biological events.

In a preferred embodiment, the invention includes a plurality of modified luciferase genes from *Renilla reniformis*. When expressed in mammalian cells, one of these polynucleotides, designated *sruc*, encodes wild type *R. reniformis* luciferase, designated SRUC, which has an amino acid sequence added so that it is secreted by mammalian cells. Advantageously, this protein is functional and its substrate is commercially available, making the secreted form of this reporter protein suitable for routine use in biological assays. When expressed in mammalian cells, another of these polynucleotides, designated *sruc3*, encodes a

modified form of wild type *R. reniformis* luciferase, designated SRUC3, which also has an amino acid sequence added so that it is secreted by mammalian cells. SRUC3 has improved bioluminescence activity and increased stability over SRUC.

Besides being used in a biological assay system alone, at least one of the polynucleotides of the present invention can be used in conjunction with the *seap* gene in a dual reporter assay system for gene expression and for other biological events in cells, including mammalian cells. Further, at least one of the polynucleotides of the present invention can be used to make stable mammalian packaging cell lines which produce retroviruses carrying the polynucleotide. Additionally, at least one of the polynucleotides of the present invention can be used as a marker gene for retroviral gene transfer experiments.

## I. PRODUCTION AND EXPRESSION OF A POLYPEPTIDE WHICH ENCODES A SECRETED FORM OF WILD TYPE *RENILLA* LUCIFERASE

### A. Construction of Plasmids Encoding Wild Type *Renilla* Luciferase Preprotein Gene

First, a plasmid containing the cDNA encoding wild type *Renilla* luciferase was constructed as follows. The gene, SEQ ID NO:1, GenBank accession number M63501, encoding wild type *Renilla* luciferase SEQ ID NO:2, was ligated as a 975 bp *Eco*RV–*Sma*I DNA fragment from plasmid pBluescript II KS(+) carrying the luciferase gene into the *Hpa*I site of plasmid pLNCX (GenBank accession number M28247), generating plasmid pLNCX-RUC. Referring now to Figure 1, there is shown a partial schematic diagram of the plasmid pLNCX-RUC, where "LTR" refers to the long terminal repeat, "*neo*" refers to the neomycin resistance gene, "CMV" refers to the cytomegalovirus promoter, "*ruc*" refers to the cDNA encoding wild type *Renilla* luciferase, and "pA" refers to the polyadenylation site. An ampicillin resistance gene and the bacterial origin of replication are not shown.

### B. Construction of Plasmids Encoding a Gene Encoding a Secreted Functional Wild Type *Renilla* Luciferase Preprotein

Next, a plasmid containing the cDNA, SEQ ID NO:3, encoding a secreted functional form of wild type *Renilla* luciferase preprotein SEQ ID NO:4, pLNCX-SRUC, was constructed as follows. First, an 87 bp DNA fragment, SEQ ID NO:5, was prepared.

SEQ ID NO:5: ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGT
CTTGCACTTGTCACAAACAGTGCACCTACTGAATTCAGCTTAA
AGATG

SEQ ID NO:5 encoded the 23 amino acid residue signal sequence of human IL-2 protein (IL2SP), GenBank accession number AJ00264, followed by a 5 amino acid linker and by the methionine start codon of wild type *Renilla* luciferase, ATG, by amplification from human embryonic kidney cell line A293 genomic DNA (Taniguchi et al., "Structure and expression of a cloned cDNA for human interleukin-2." *Nature* 302:305-310 (1983)), using the polymerase chain reaction (PCR) with primers SEQ ID NO:6, and SEQ ID NO:7.

SEQ ID NO:6 TTTGAATTCATGTACAGGATGCAACTCCT

SEQ ID NO:7 TTTGAATTCAGTAGTGCACTGTTTGTGAC

PCR was performed using Pwo polymerase (Boehringer-Mannheim, Mannheim, Germany) and thermocycling for 30 cycles at 94°C for 15 seconds and 50°C for 30 seconds.

The PCR fragment was cloned directly into vector pGEMT (Promega, Madison, WI, US), excised using the *Eco*RI sites introduced by both primers, and ligated 9 bp upstream of the *Renilla* luciferase gene in plasmid pLXSN-RUC. (Liu et al., "Secretion of functional *Renilla reniformis* luciferase by mammalian cells." *Gene* 203:141-148 (1997)). The correct sequences of the PCR product and of the in-frame fusion of this product with wild type *Renilla* luciferase gene were confirmed by automated DNA sequencing. The DNA sequence encoding the IL2SP-*Renilla* luciferase protein fusion was amplified using primers SEQ ID NO:6, and SEQ ID NO:8.

SEQ ID NO:8 TTTCCCGGGAAAAATGTAAATAAAAAACCA

The PCR product carrying the gene encoding the IL2SP–*Renilla* luciferase protein fusion (*sruc*), was then cloned into the plasmid pGEMT and subcloned as a *Ksp*I–*Sma*I fragment into the *Hpa*I site of retroviral vector pLNCX, under transcriptional control of the CMV promoter, generating plasmid pLNCX-SRUC for mammalian cell expression studies, and for future packaging into retroviral particles for introduction into animal model system.

Referring now to Figure 2, there is shown a partial schematic diagram of the plasmid pLNCX-SRUC, showing the protein fusion junction, where "LTR" refers to the long terminal repeat, "*neo*" refers to the neomycin resistance gene, "CMV" refers to the cytomegalovirus promoter, "*ruc*" refers to the cDNA encoding wild type *Renilla* luciferase, and "pA" refers to the polyadenylation site. An ampicillin resistance gene and the bacterial origin of replication are not shown. The putative site of cleavage of the signal peptidase is shown by the arrow between residues 20 and 21.

**C. Transfection of Cultured Mammalian Cells with Plasmids Encoding the Wild Type *Renilla* Luciferase Protein Gene and Secreted Wild Type *Renilla* Luciferase Preprotein Gene**

Simian COS-7 cells were cultured and transfected with the plasmids pLNCX, pLNCX-RUC, or pLNCX-SRUC as follows. First, the mammalian cells were grown in 100 mm tissue culture plates containing 10 ml DMEM medium (Sigma Chemical, St. Louis, MO, US) supplemented with 10% fetal bovine serum (FBS) (Biowhittaker, Walkersville, MD, US). The medium was changed 3 hours prior to transfection when cells were 75% confluent.

Each plate of the cultured mammalian cells was transfected with the 40 μg of plasmid DNA per plate using the ProFection Calcium Phosphate System (Promega), according to the manufacturer's instructions. Six hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS). Then, 10 ml of DMEM medium and 10% FBS were added to each plate. No differences in cell morphology or cell growth were observed between cell expressing the wild type *Renilla* luciferase gene, and cells expressing the *sruc* gene, indicating that presence of the *sruc* gene does not affect cell growth or morphology when compared to cells expressing the *ruc* gene.

**D. Measurement of *Renilla* Luciferase Stability in Cell Culture Media and in Whole Blood**

The stability of *Renilla* luciferase in cell culture media was determined as follows. Three nanograms of purified *Renilla* luciferase (isolated from an overexpressing *Escherichia coli* strain and lacking IL2SP) was added to a 100 mm plate containing COS-7 cells growing at 75% confluency in 10 ml DMEM supplemented with 10% FBS, and another three nanograms of purified *Renilla* luciferase was added to a 100 mm plate containing COS-7 cells growing at 75% confluency in 10 ml QBSF 51 supplemented with 1% FBS. The plates were incubated at 37°C in 5% $CO_2$, and a 200 μl aliquot of medium was taken at regular time intervals for bioluminescence assay.

The stability of *Renilla* luciferase in whole blood was determined as follows. Three nanograms of the same isolated *Renilla* luciferase was added to 700 μl of freshly obtained whole hamster blood containing 50 μl of a heparin solution (1000 units/ml, from Elkins-Sinn), and placed at 37°C. A 10 μl aliquot was taken at regular time intervals, and diluted into 200 μl assay buffer for bioluminescence assay.

Referring now to Figure 3, there is shown a graph of the results of these assays. As can be seen, isolated *Renilla* luciferase lacking IL2SP added to DMEM medium supplemented with 10% FBS (circles), had a half-life of approximately 50 minutes. Isolated *Renilla* luciferase lacking IL2SP added to QBSF 51 medium supplemented with 1% FBS (diamonds) had a half-life of approximately 57 minutes, indicating that luciferase stability did not vary significantly between these two media. Finally, isolated *Renilla* luciferase lacking IL2SP added to whole blood (triangles) had a half-life of approximately 36 minutes, an advantageously short half-life for assays to be perform in closed systems. Relative light units (RLU) are shown as per second, per 200 $\mu$l medium.

**E. Bioluminescence Assays of Luciferase Activity in Culture Media Containing Secreted *Renilla* Luciferase and in Cell Lysates of Transfected Mammalian Cells**

Bioluminescence assays of *Renilla* luciferase activity was measured from both cell lysates and from the culture media of the mammalian cells transfected with plasmids pLNCX, pLNCX-RUC, or pLNCX-SRUC. In each case, cells from one plate were harvested by scraping in assay buffer containing 0.5 M NaCl, 1 mM EDTA, and 100 mM potassium phosphate, at pH 7.4, 48 hours after transfection, washed once with the assay buffer, resuspended in 1 ml of assay buffer, and then sonicated on ice twice for 10 seconds. Then, 500 $\mu$l of cell lysates in each case were assayed for bioluminescence for 10 seconds using a Turner TD-20e luminometer after rapid injection to ensure even mixing of 500 $\mu$l of 1 $\mu$M coelenterazine hcp (Molecular Probes, Eugene, OR, US).

Referring now to Figures 4 and 5, there are shown a bar graph of the *Renilla* luciferase activity in cell lysates and in cell culture media, respectively. Light emission was measured for cells transfected with (1) pLNCX, (2) pLNCX-RUC, or (3) pLNCX-SRUC. Results obtained from six individual transfections are shown. Relative light units (RLU) are show as per second, per 50 $\mu$g total protein in Figure 4 and as per second, per 200 $\mu$l medium in Figure 5.

As can be seen in Figure 4, *Renilla* luciferase activity measured from lysates of cells expressing the *Renilla* luciferase gene construct was approximately 15-times higher than that measured from lysates of cells expressing the *sruc* gene construct. By contrast, as can be seen in Figure 5, only cell culture media in which cells expressing the IL2SP-*Renilla* luciferase gene fusion (*sruc*) were growing (SRUC medium) contained significant levels of

*Renilla* luciferase activity. Luciferase activity measured in the SRUC medium was not the result of cell lysis, since 10 ml of SRUC medium (DMEM supplemented with 10% FBS) from a 100% confluent 100 mm dish contained, on average, 6000 relative light units (RLU) 48 hours after transfection, while cell lysates from the same culture dish contained, on average, only 600 RLU.

Additionally, the time course of appearance of *Renilla* luciferase activity in cell culture media was assayed at regular time intervals using the same method on 200 $\mu$l aliquots of culture media taken from dishes containing growing the transfected cells. 200 $\mu$l of fresh media was added each time to the plates as replacement for the aliquot taken. In one plate containing cells transfected with plasmid pLNCX-SRUC were grown in DMEM medium supplemented with 10% FBS for the first 42 hours, and then grown in QBSF 51 (Sigma) medium supplemented with1% FBS to investigate the effects of medium composition on accumulation of functional secreted *Renilla* luciferase.

Referring now to Figure 6, there is shown a graph of the results of the assays of time course of appearance of *Renilla luciferase* activity in cell culture media of mammalian cells transfected with plasmid pLNCX-SRUC and grown in DMEM medium supplemented with 10% FBS (circles); transfected with plasmid pLNCX-SRUC and grown in DMEM medium supplemented with 10% FBS for the first 42 hours, and then grown in QBSF 51 medium supplemented with 1% FBS (triangles); transfected with plasmid pLNCX-RUC and grown in DMEM medium supplemented with 10% FBS (diamonds); and transfected with vector only and grown in DMEM medium supplemented with 10% FBS (squares). Relative light units (RLU) are shown as per second, per 200 $\mu$l medium.

As can be seen, *Renilla* luciferase activity could be detected in cell culture medium less than 10 hours after cell transfection, but only with cells transfected with plasmid pLNCX-SRUC (circles and triangles). By contrast, medium taken from plates containing cells transfected with plasmid pLNCX-RUC did not show detectable *Renilla* luciferase activity (diamonds). In the case of cells transfected with pLNCX-SRUC, luciferase activity increased over time until it reached a plateau 48 hours after transfection. Significantly, while the rate of cell growth was reduced by replacing the medium with QBSF 51 medium supplemented with 1% FBS, luciferase activity continued to increase throughout the study period of 80 hours after transfection (triangles). These results indicate that the *Renilla*

luciferase protein was secreted in cells transfected with pLNCX-SRUC because effect occurred even though the difference in media did not greatly affect luciferase stability (Figure 3).

**F. Immunoblot Analysis of the Presence of *Renilla* Luciferase in Transfected Cells and in Cell Culture Media**

The presence of *Renilla* luciferase in both lysates of transfected cell and in cell culture media was investigated using immunoblot analysis after protein fractionation with SDS-PAGE. For immunoblot analysis of intracellular *Renilla* luciferase, mammalian COS-7 cells from one plate were washed twice with cold PBS 48 hours after transfection, and harvested by scraping into 100 $\mu$l 2 x gel-loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue). After complete lysis by sonication, samples were boiled for 3 minutes, centrifuged at 10000 x *g* for 10 minutes to pellet debris, and 20 $\mu$l of each sample was loaded on a 12% SDS-polyacrylamide gel. Fractionated proteins were then transferred by electroblotting onto a nylon membrane, reacted with a monoclonal antibody raised against *Renilla* luciferase, and detected using chemiluminescence. Protein amounts were determined by comparison with a 10 ng of isolated *Renilla* luciferase protein present on the same blot as a control, using a BioImage Whole Band Analyzer (Genomic Solutions Inc., Ann Arbor, MI US).

For immunoblot analysis of secreted *Renilla* luciferase, 3 ml aliquots of cell culture media (QBSF 51 supplemented with 1% FBS) were taken 48 hours after transfection of COS-7 cells and centrifuged at 10,000 x *g* to pellet cell debris. The aliquots were concentrated down to 0.5 ml using a centriprep concentrator (Amicon, Beverly, MA, US) with a molecular weight cut off of 15,000. *Renilla* luciferase present in the concentrates was immunoprecipitated using the Protein A Immunoprecipitation Kit ((Boehringer-Mannheim, Mannheim, Germany) and monoclonal antibody raised against *Renilla* luciferase, and immunoanalyzed after SDS-PAGE using the same monoclonal antibody.

Referring now to Figures 7 and 8, there are shown immunoblots of total cell lysates, and of immunoprecipitated cell culture media, respectively, using *Renilla* luciferase monoclonal antibody. Lane 1 contained 10 ng isolated *Renilla* luciferase as a control; lane 2 had the mammalian cells that were transfected with plasmid pLNCX-SRUC; lane 3 had the mammalian cells that were transfected with plasmid pLNCX-RUC; and lane 4 had the

mammalian cells that were transfected with vector only. Protein amounts were determine by densitometry after comparison with isolated *Renilla* luciferase protein, lane 1.

Referring now to Figure 7, protein amounts were calculated to be 0.9 ng for the cells transfected with plasmid pLNCX-SRUC, lane 2, and 1.7 ng for the cells transfected with plasmid pLNCX-RUC, lane 3. The decrease in the amount of luciferase in the cells transfected with plasmid pLNCX-SRUC is an indication that *sruc* encoded a secreted product which was, therefore, not accumulating in the cytosol. Further, these results indicated that the 15-times increase in luciferase activity measured from lysates of cells expressing the *Renilla* luciferase gene construct as compared to that measured from lysates of cells expressing the *sruc* gene construct, Figure 3, could not be explained by relative amount of *Renilla* luciferase protein alone.

By contrast, Figure 8 shows that only the cell culture media of cells transfected with pLNCX-SRUC, lane 2, demonstrated the presence of *Renilla* luciferase. A protein band of lower molecular weight is also present in lane 2 and faintly in lane 3 and probably represents degradation products of *Renilla* luciferase.

## II. USE OF THE SECRETED FORM OF WILD TYPE *RENILLA* LUCIFERASE AS A REPORTER PROTEIN

According to one embodiment of the present invention, there is provided a method of using the secreted form of wild type *R. reniformis* luciferase as a reporter protein in a biological assay to study a variety of biological events. The method includes introducing the gene encoding the secreted *Renilla* luciferase to a cell type or tissue using techniques as will be understood by those with skill with reference to the disclosure herein, such as calcium phosphate co-precipitation, electroporation, lipofection, or naked DNA injection. Samples from the solution surrounding the cells, such as cell culture medium or body fluid, expressing the secreted *Renilla* luciferase gene are then analyzed. Analysis can include placing the sample solution in an assay buffer, such as 0.5 M NaCl, 1 mM EDTA, and 100 mM potassium phosphate at pH 7.4, and recording light emission for a short period of time (5–10 seconds) after addition of the substrate solution. The method can further include measuring the number of accumulated photons over longer periods of time which increases the sensitivity of the assay because *Renilla* luciferase catalyzes a "glow-type" of light emission.

The method of using the secreted form of wild type *R. reniformis* luciferase as a reporter protein, according to the present invention, advantageously has a sensitivity in the order of 100 fg, which is three orders of magnitude more sensitive than the assay for secreted human growth hormone. Further advantageously, the method of the present invention is more rapid (less than 1 minute) compared to the secreted AP assay (more than 45 minutes) or to the secreted apoaeqorin assay (more than 2 hours). Also, the method is advantageous as a biological assay in mammals because *Renilla* luciferase does not have a biological function having an effect on metabolism or development of the animal, such as human growth hormone can have when used in a biological assay.

Additionally advantageously, the method can be used where previously secreted protein can not be removed before the beginning of a time-course study because of the short half-life of *Renilla* luciferase. For example, secreted *Renilla* luciferase can be used in a closed system such as monitoring gene expression in animal model systems, including studies of transcriptional activities during animal development, during host-pathogen interactions, or after gene transfer for gene therapy purposes, without killing the animal.

## III. PRODUCTION AND EXPRESSION OF POLYPEPTIDE WHICH ENCODES A SECRETED MODIFIED FORM OF WILD TYPE *RENILLA* LUCIFERASE

### A. Construction of Plasmids Encoding the Secreted Wild Type *Renilla* Luciferase Preprotein Gene and Site-specific Mutagenesis of the Gene

Plasmids encoding the secreted wild type *Renilla* luciferase preprotein gene were constructed and site-specific mutagenesis of the gene using these plasmids were performed as follows. First, the *sruc* gene, SEQ ID NO:3, encoding the preprotein form of the secreted *Renilla* luciferase, SEQ ID NO:4, was excised from plasmid pLNCX-SRUC, shown in Figure 2, as a 1.1 kb *Hind*III-*Cla*I DNA fragment with the *Cla*I site blunt-ended with Klenow enzyme, and was ligated into the HindIII-SmaI sites of plasmid pBluescript KS(+)II (Stratagene, San Diego, CA, US). The *sruc* gene, SEQ ID NO:3, was then subcloned as a *Sal*I-*Xba*I DNA fragment into plasmid vector pND2 (a gift from Gary Rhodes and Robert Malone, unpublished data), under the transcriptional control of the cytomegalovirus promoter, generating plasmid pND2-SRUC, all according to techniques known to those with skill in the art.

Plasmid pND2-SRUC then served as a template for site-directed mutagenesis of the

gene, SEQ ID NO:3, encoding the pre-protein form of the secreted *Renilla* luciferase, SEQ ID NO:4. In summary, the Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol to introduce isolated mutations in SEQ ID NO:3 which caused cysteine to alanine substitutions at positions 52, 101, and 152, and at all three positions 52, 101 and 152, in the pre-protein form of secreted *Renilla* luciferase, SEQ ID NO:4; where the mutated genes, *sruc1*, SEQ ID NO:9, coded for SRUC1, SEQ ID NO:10, having a cysteine to alanine substitution at position 52; *sruc2*, SEQ ID NO:11, coded for SRUC2, SEQ ID NO:12, having a cysteine to alanine substitution at position 101; *sruc3*, SEQ ID NO:13, coded for SRUC3, SEQ ID NO:14, having a cysteine to alanine substitution at position 152; and *sruc4*, SEQ ID NO:15, coded for SRUC4, SEQ ID NO:16, having cysteine to alanine substitutions at position 52, 101 and 152; respectively, generating plasmids pND2-SRUC1, pND2-SRUC2, pND2-SRUC3 and pND2-SRUC4, respectively. No substitution was made for the cysteine residue present in the IL-2 leader sequence.

Referring now to Figure 9, there are shown schematic diagrams of the amino acid sequences of the secreted *Renilla* luciferases pre-proteins, SRUC1, SEQ ID NO:10; SRUC2, SEQ ID NO:12; SRUC3, SEQ ID NO:14; and SRUC4, SEQ ID NO:16, generated using these techniques, compared with SRUC, SEQ ID NO:4. As can be seen, each sequence was 342 amino acid residues in length and was a fusion of the twenty-three amino acid residues of the human interleukin-2 leader peptide, the five amino acid linker Glu Phe Ser Leu and Lys, and Met, and the 311 amino acid residues of wild type *Renilla* luciferase pre-protein sequence, SEQ ID NO:2. The first sequence, SRUC, SEQ ID NO:4, contained the complete native *Renilla* luciferase pre-protein sequence. The second sequence, SRUC1, SEQ ID NO:10, contained a single cysteine to alanine substitution at position 52. The third sequence, SRUC2, SEQ ID NO:12; contained a single cysteine to alanine substitution at position 101. The fourth sequence, SRUC3, SEQ ID NO:14; contained a single cysteine to alanine substitution at position 152. The fifth sequence, SRUC4, SEQ ID NO:16; contained three cysteine to alanine substitutions at positions 52, 101 and 152, SEQ ID NO:4.

**B. Transfection of Cultured Mammalian Cells with Plasmids Encoding the Secreted Wild Type *Renilla* Luciferase Preprotein Gene and Modified Forms of the Secreted Wild Type *Renilla* Luciferase Preprotein Gene**

Simian COS-7 cells were cultured and transfected with the plasmids pND2-SRUC1,

pND2-SRUC2, pND2-SRUC3 and pND2-SRUC4 as follows. First, some of the mammalian cells were grown in 100 mm tissue culture plates containing 10 ml DMEM medium (Sigma) supplemented with 10% fetal bovine serum (Biowhittaker), and others were grown in Cellgro complete serum free medium (Mediatech, Herndon, VA, US) and 1% fetal bovine serum. Each plate of the cultured mammalian cells were transfected with the 30 $\mu$g of plasmid which comprised 28 $\mu$g of one of the plasmids encoding a secreted *Renilla* luciferase preprotein gene for experimental purpose and 2 $\mu$g of firefly luciferase plasmid for normalization between experiments using the ProFection Calcium Phosphate System (Promega), according to the manufacturer's instructions. Fifteen hours after transfection, the cells were then washed twice with phosphate-buffered saline. Then, 10 ml of DMEM medium supplemented with 10% FBS was added to the cells grown in this medium and Cellgro complete serum free medium supplemented with 1% FBS was added to the cells grown in this medium.

**C. Immunoblot Analysis of the Presence of Secreted *Renilla* Luciferase and Modified Forms of the Secreted *Renilla* Luciferase in Transfected Cells**

An immunoblot analysis of the secreted *Renilla* luciferase preprotein and the modified forms of the secreted *Renilla* luciferase preprotein in lysates of transfected mammalian cells was performed as previously described to determine if the modified secreted *Renilla* luciferase preproteins retained their full length intracellularly as compared to wild type *Renilla* luciferase. Briefly, 25 ug of total cell lysates were fractionated using SDS-PAGE, transferred to a membrane, and reacted with rabbit polyclonal antibodies raised against wild-type *Renilla* luciferase. Referring now to Figure 10, there is shown an immunoblot analysis for the presence of *Renilla* luciferase in total cell lysates of mammalian cells transfected with expressing genes encoding SRUC (lane 1), SRUC1 (lane 2), SRUC2 (lane 3), SRUC3 (lane 4), and SRUC4 (lane 5), and isolated from *Escherichia coli* expressing the *Renilla* luciferase cDNA as control (lane 6). As can be seen, lysates of all of the mammalian cells demonstrate expression of a polypeptide having a weight of about 35.5 kDa, indicating that all of the modified secreted *Renilla* luciferase preproteins retained their full length.

**D. Bioluminescence Assays of Luciferase Activity in Culture Media Containing Secreted *Renilla* Luciferases and in Cell Lysates of Transfected Mammalian Cells**

Bioluminescence assays of luciferase activity were performed on cell lysates of mammalian cells transfected with plasmids pND2-SRUC, pND2-SRUC1, pND2-SRUC2,

pND2-SRUC3 and pND2-SRUC4; and on culture media (serum free medium supplemented with 1% FBS) containing secreted *Renilla* luciferases from mammalian cells transfected with plasmids pND2-SRUC, pND2-SRUC1, pND2-SRUC2, pND2-SRUC3, and pND2-SRUC4 using a Turner TD-20e luminometer 48 hours after transfection. 200 $\mu$l aliquots of cell culture media containing transfected cells with plasmids pND2-SRUC, pND2-SRUC1, pND2-SRUC2, and pND2-SRUC4 were used for each assay. In order to keep the light emission readings within the scale of the luminometer, however, only 2 $\mu$l aliquots of cell culture media containing transfected cells with plasmids pND2-SRUC3 diluted into 198 $\mu$l of culture medium were used for the assay of SRUC3 activity, and then the number of relative light units (RLU) obtained was multiplied by 100. Unmodified coelenterazine was used as a substrate (catalog # C-2944, Molecular Probes).

Referring now to Figure 11, there is shown a bar graph depicting the results of the measured *Renilla* luciferase bioluminescence activity in mammalian cell lysates for cells transfected with *sruc*1 (lane 1), *sruc*2 (lane 2), *sruc*3 (lane3), *sruc*4 (lane 4) and *sruc* (lane 5). As can be seen, mammalian cells transfected with *sruc*1 demonstrated about half of the *Renilla* luciferase activity as mammalian cells transfected with *sruc*. By contrast, mammalian cells transfected with *sruc*3 demonstrated about three times the *Renilla* luciferase activity as mammalian cells transfected with *sruc*. Mammalian cells transfected with either *sruc*2 or *sruc*4 did not demonstrate significant intracellular bioluminescence activities.

Referring now to Figure 12, there is shown a bar graph depicting the results of the measured *Renilla* luciferase bioluminescence activity in culture media of the same cells transfected with SRUC1 (lane 1), SRUC2 (lane 2), SRUC3 (lane3), SRUC4 (lane 4) and SRUC (lane 5). As can be seen, the culture media of mammalian cells transfected with SRUC3 exhibited a bioluminescence activity of approximately 100 times the culture media of mammalian cells transfected with SRUC. The culture media of mammalian cells transfected with either SRUC1, SRUC2 or SRUC4 did not show significant bioluminescence activity.

Further, the time course of appearance of *Renilla* luciferase activity was assayed at regular time intervals in cell culture media (Cellgro complete serum free medium supplemented with 1% FBS) containing secreted *Renilla* luciferases from mammalian cells transfected with plasmids pND2-SRUC, pND2-SRUC1, pND2-SRUC2, pND2-SRUC3, and pND2-SRUC4 over 90 hours after transfection. Referring now to Figure 13, there is show a

graph of the results of the assays of time course of appearance of *Renilla luciferase* activity in cell culture media. As can be seen, culture media containing secreted *Renilla* luciferases from mammalian cells transfected with plasmids pND2-SRUC (closed circles) increased for up to about 30 hours after transfection and then decreased steadily towards baseline. By contrast, culture media containing secreted *Renilla* luciferases from mammalian cells transfected with plasmids pND2-SRUC3 (closed triangles) peaked at about 30 hours but remained relatively high through the end of the assay period. Culture media containing secreted *Renilla* luciferases from mammalian cells transfected with plasmids pND2-SRUC1 (x's) and pND2-SRUC2 (closed squares) did not show significant activity during the entire 90 hour assay period.

**E. Determination of Cell Culture Medium Dependency of Secreted *Renilla* Luciferase Activity:**

Next, a determination was made on whether SRUC3 luciferase activity was dependent on the type of culture medium in which cells secreting that protein were growing as follow. One group of COS-7 cells were transfected with plasmid pND2-SRUC3 grown in DMEM medium supplemented with 10% FBS for 48 hours, and a second group of COS-7 cells were transfected with plasmid pND2-SRUC3 grown in serum free medium supplemented with 1% FBS for 48 hour. *Renilla* luciferase activity was measured in both cell lysates and culture media.

Referring now to Figure 14, there are shown bar graphs of the *Renilla* luciferase activity for cell lysates (cross hatching up to the right) and culture medium (cross hatching up to the left) of cells grown in DMEM supplemented with 10% FBS (left most bars) and cells grown serum free medium (SFM) supplemented with 1% FBS (right most bars). As can be seen, lysates and culture medium of cells grown in DMEM supplemented with 10% FBS showed a two fold and a more than five fold reduction in light emission, respectively, when compared to lysates and culture medium of cells grown serum free medium supplemented with 1% FBS.

**F. Measurement of Secreted *Renilla* Luciferase Stability in Cell Culture Media**

In order to determine whether the observed increased bioluminescence activity of SRUC3 compared with SRUC when secreted by mammalian cells, above, was due to increased stability of the SRUC3 luciferase compared with SRUC, the activities of already

secreted SRUC and SRUC3 luciferases were measured over time at 37°C in serum free medium supplemented with 1% FBS. Referring now to Figure 15, there is shown a graph of the results where the closed diamonds represent bioluminescence activity of SRUC secreted by mammalian cells, and the closed squares represent bioluminescence activity of SRUC secreted by mammalian cells. From the data obtained, the half-life of SRUC and SRUC3 was calculated to be 14 hours and 82 hours, respectively. These stability measurements confirmed that the observed increased bioluminescence activity of SRUC3 compared with SRUC when secreted by mammalian cells, above, was at least in part due to increased stability of the SRUC3 luciferase compared with SRUC.

## IV. USE OF SRUC3 AND SEAP GENES IN DUAL REPORTER SYSTEM

### A. Construction of Plasmids Encoding *sruc3* and *seap* Genes

First, a plasmid containing the *sruc3* gene was reconstructed into retroviral vector pLNCX by cloning a *Bgl*II- *Xba*I DNA fragment from plasmid pND2-SRUC3 into plasmid pLNCX-SRUC, generating plasmid pLNCX-SRUC3. The *seap* gene (Clontech, Palo Alto, CA, US), which encodes SEAP protein was cloned into pLNCX (Miller, et al., "*Improved retroviral vectors for gene transfer and expression.*" *Biotechniques* October; 7(9):980-2, 984-6, 989-90 (1989)) as a *Hind*III-*Bgl*I DNA fragment, generating plasmid pLNCX-SEAP, both according to techniques known to those with skill in the art. Both genes were under transcriptional control of the cytomegalovirus promoter.

### B. Co-Transfection of Cultured Mammalian Cells with Plasmids pLNCX-SRUC3 and pLNCX-SEAP

Simian COS-7 were grown in DMEM medium supplemented with 10% fetal bovine serum and three culture dishes of cells were transiently co-transfected with plasmids pLNCX-SRUC3 and pLNCX-SEAP using the ProFection Calcium Phosphate System (Promega) according to the manufacturer's instructions in the following ratios: 5 μg sruc3 + 15 μg seap (dish 1); 10 μg sruc3+ 10 μg seap (dish 2); and 15 μg sruc3 + 5 μg seap (dish 3).

### C. Bioluminescence Assays of Luciferase Activity and Chemiluminescent Assays of Alkaline Phosphatase Activity in Culture Media Containing Secreted *Renilla* Luciferase and Secreted Alkaline Phosphatase

The *Renilla* luciferase activity assay was performed as described above, and the alkaline phosphatase assay was performed according to the manufacturer's protocol

(Clontech) 48 hours after co-transfection from centrifuged cell culture media. Light emission was measured in relative light units (RLU) using a Turner TD-20e luminometer. The *Renilla* luciferase and alkaline phosphatase assays were done with 50 μl and 1 μl of media, respectively, and SEAP measurements were multiplied by a correction factor of 50.

Referring now to Figures 16 and 17, there are shown bar graphs of the results of the bioluminescence assays of luciferase activity, and the chemiluminescent assays of alkaline phosphatase activity; respectively. As can be seen, the *sruc3* and *seap* genes were co-expressed in mammalian cells, indicating that these genes can be used in a dual reporter assay system. This dual reporter assay system based on secreted *Renilla* luciferase and an additional secreted light emitting protein is simple to perform, since no cell harvesting and lysis are involved. Other genes encoding light secreting proteins which can be used *with* secreted Renilla luciferase include secreted alkaline phosphatase, and *Vargula* luciferase. Further, the light emission catalyzed by SRUC3 increased proportionally with increased amounts of DNA, indicating that sensitivity of the SRUC3 assay could be increased relative to the other light emitting protein assay in mammalian cells.

## V. CONSTRUCTION OF STABLE MAMMALIAN PACKAGING CELL LINES WHICH PRODUCE RETROVIRUSES CARRYING THE *SRUC3* GENE

A stable mammalian packaging cell lines which produced retroviruses carrying the *sruc3* gene was produced as follows. First, ecotropic GP+E86 retroviral packaging cells were transfected with 20 μg of plasmid pLNCX-SRUC3 using the ProFection Calcium Phosphate System (Promega) according to the manufacturer's instructions. Then, supernatants obtained from stable GP+E86 cell lines transfected with pLNCX-SRUC3 were used for transduction of amphotropic packaging cell line PA317. Sixty-one stable transformants were obtained after selection in presence of G418 and *Renilla* luciferase activity was measured in their culture media.

Referring now to Figure 18, there is shown a chart of the range of *Renilla* luciferase activities measured in 100 μl aliquots of media of 61 stable PA317 packaging cell lines transduced with retroviruses carrying the *sruc3* gene. As can be seen, 85% of the isolated PA317 colonies demonstrated some level of *Renilla* luciferase activity in their culture media and 8% of the isolated PA317 colonies showed high levels of *Renilla* luciferase activity in their culture media (greater than 1000 RLU). These results indicate that the *sruc3* gene can

be stably expressed at high levels in mammalian cells.

All documents cited herein are incorporated herein by reference in their entirety.

Although the present invention has been discussed in considerable detail with reference to certain preferred embodiments, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of preferred embodiments contained herein.

WE CLAIM:

1. A polynucleotide encoding a secreted *Renilla* luciferase.

2. The polynucleotide of claim 1, comprising a nucleotide sequence according to SEQ ID NO:3.

3. The polynucleotide of claim 1, comprising a nucleotide sequence according to SEQ ID NO:13.

4. A polynucleotide comprising a nucleotide sequence according to SEQ ID NO:3.

5. A polynucleotide comprising a nucleotide sequence according to SEQ ID NO:13.

6. A secreted *Renilla* luciferase.

7. The polypeptide of claim 6, comprising an amino acid sequence according to SEQ ID NO:4.

8. The polypeptide of claim 6, comprising an amino acid sequence according to SEQ ID NO:14.

9. A polypeptide comprising an amino acid sequence according to SEQ ID NO:4.

10. A polypeptide comprising an amino acid sequence according to SEQ ID NO:14.

11. A method of performing a biological assay comprising providing a polynucleotide encoding a secreted *Renilla* luciferase.

12. The method of claim 11, where the polynucleotide provided comprises a nucleotide sequence according to SEQ ID NO:3.

13. The method of claim 11, where the polynucleotide provided comprises a nucleotide sequence according to SEQ ID NO:13.

14. The method of performing a biological assay of claim 11, additionally comprising transfecting a host cell with the polynucleotide.

15. The method of performing a biological assay of claim 14, where the host cell in the transfecting step is a mammalian cell.

16. The method of performing a biological assay of claim 14, where the host cell is growing in a culture media, and where the method additionally comprises detecting light emission from the *Renilla* luciferase coded by the polynucleotide that has been secreted in the culture media.

17. The method of claim 16, where the *Renilla* luciferase detected comprises an amino acid sequence according to SEQ ID NO:4.

18. The method of claim 16, where the *Renilla* luciferase detected comprises an amino acid sequence according to SEQ ID NO:14.

19. The method of performing a biological assay of claim 14, additionally comprising transfecting the host cell with a second polynucleotide encoding a second light emitting protein.

20. The method of performing a biological assay of claim 19, where the second light emitting protein in the coded by the second polynucleotide is *seap*.

21. The method of performing a biological assay of claim 19, where the host cell is growing in a culture media, and where the method additionally comprises detecting light emission from the second light emitting protein coded by the second polynucleotide that has been secreted in the culture media.

22. A method of performing a biological assay comprising providing the polynucleotide of claim 1.

23. The method of claim 22, additionally comprising providing a second polynucleotide encoding a light emitting protein.

24. The method of claim 23, additionally comprising providing *seap*.

25. A method of performing a biological assay comprising detecting light emission from a polypeptide encoded by the polynucleotide of claim 1.

26. The method of claim 25, additionally comprising detecting light emission from a second polypeptide.

27. The method of claim 26, where the second polypeptide is SEAP.

28. A plasmid containing the polynucleotide of claim 1.

29. A vector containing the polynucleotide of claim 1.

30. A host cell transfected with the polynucleotide of claim 1.

31. A mammalian host cell transfected with the polynucleotide of claim 1.

32. A kit for performing a biological assay, comprising the polynucleotide of claim 1.

33. The kit of claim 32, additionally comprising a second polynucleotide.

34. The kit of claim 33, where the second polynucleotide is *seap*.

35. A kit for performing the method of claim 11, comprising a polynucleotide encoding a secreted *Renilla* luciferase.

36. The kit of claim 35, additionally comprising a second polynucleotide.

37. The kit of claim 36, where the second polynucleotide is *seap*.

38. A stable mammalian packaging cell line which produces retroviruses carrying a polynucleotide encoding a secreted *Renilla* luciferase.

39. The stable mammalian packaging cell line of claim 38, where the polynucleotide encodes the SEQ ID NO:14.

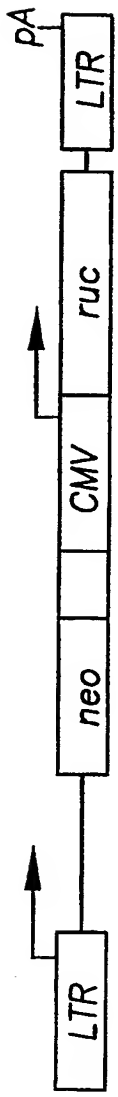


FIG. 1

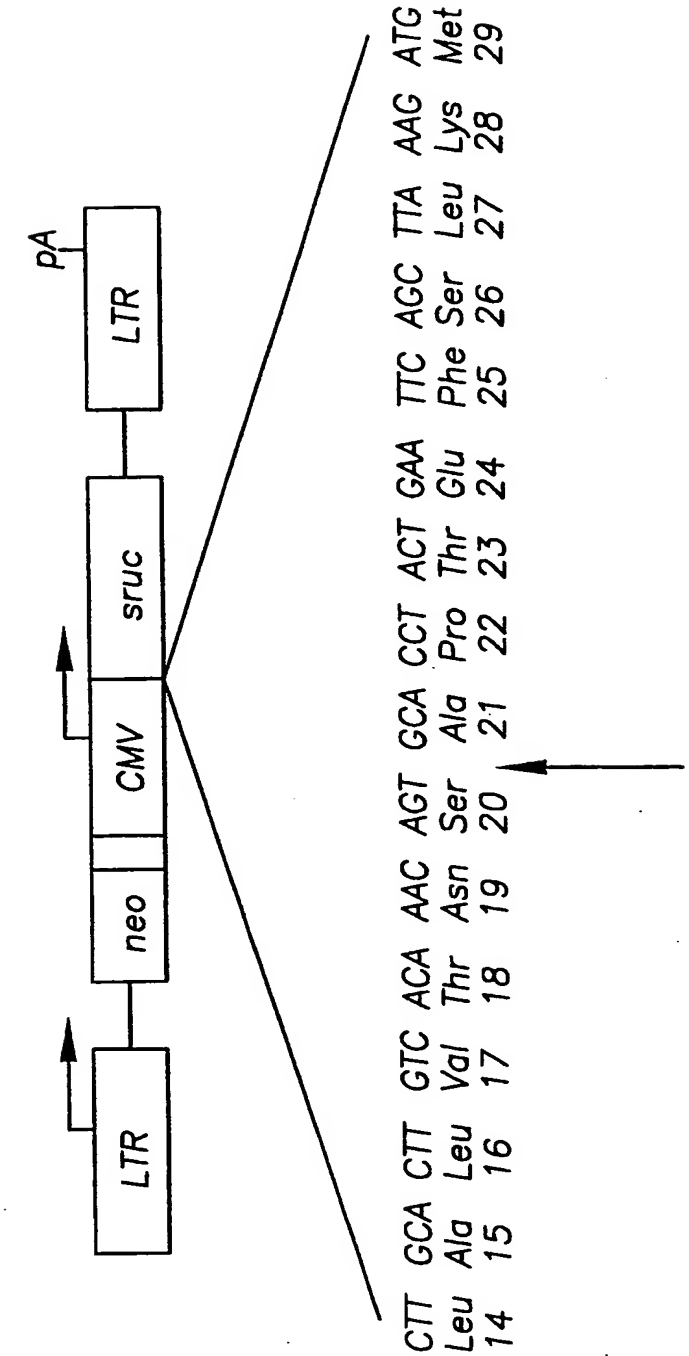
pA
LTR
sruc
CMV
neo
LTR
CTT GCA CTT GTC ACA AAC AGT GCA CCT ACT GAA TTC AGC TTA AAG ATG
Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met
14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

FIG.2

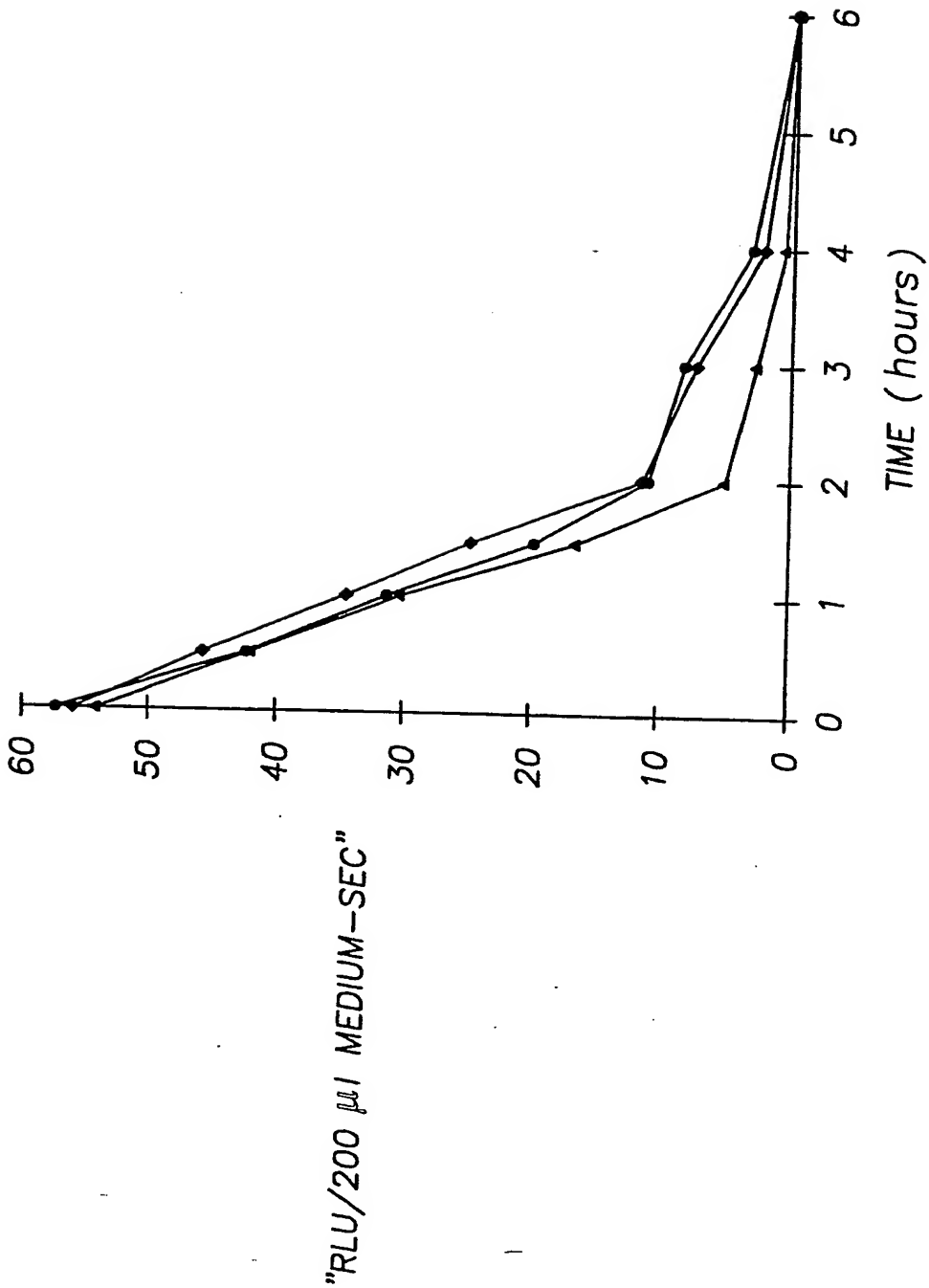


FIG.3

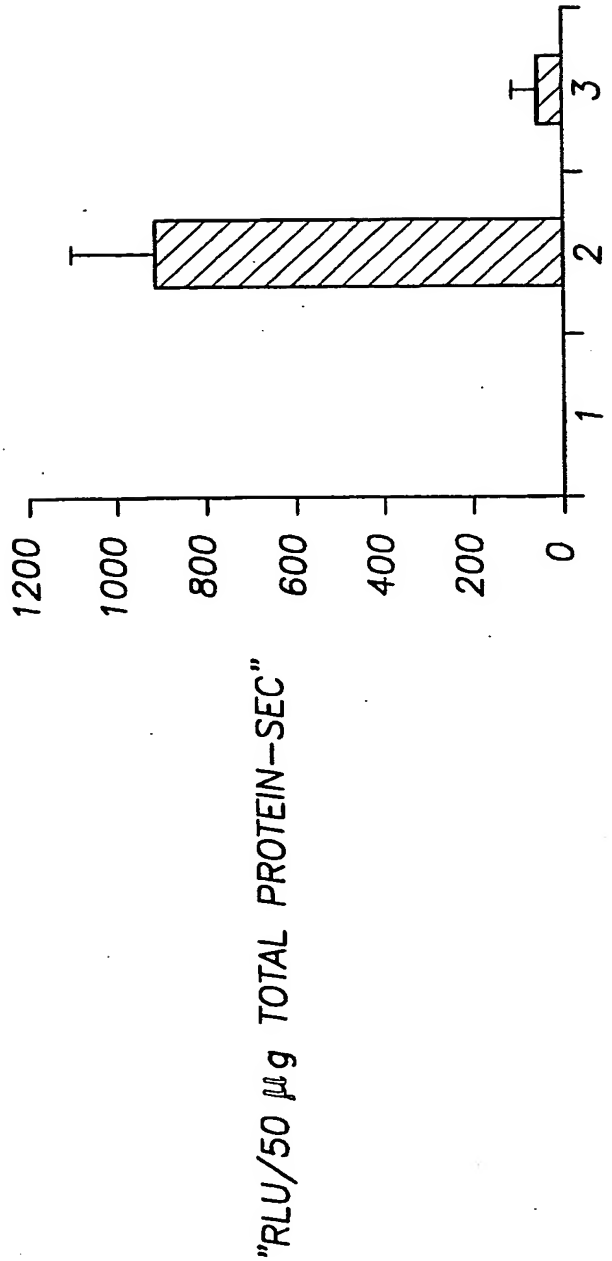


FIG.4

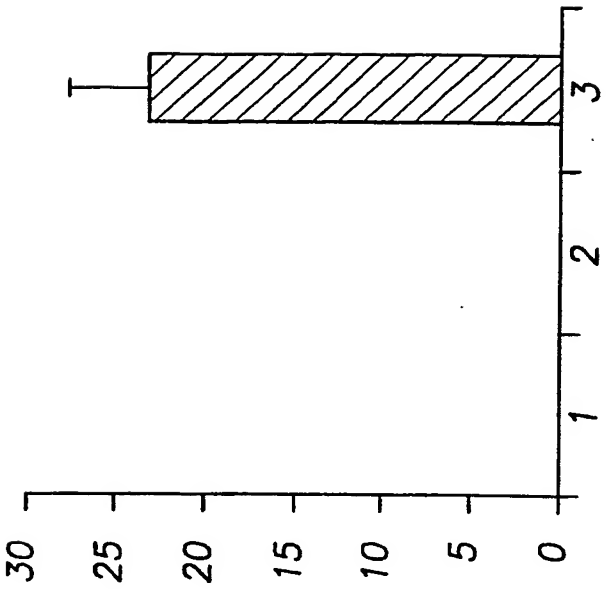


FIG.5

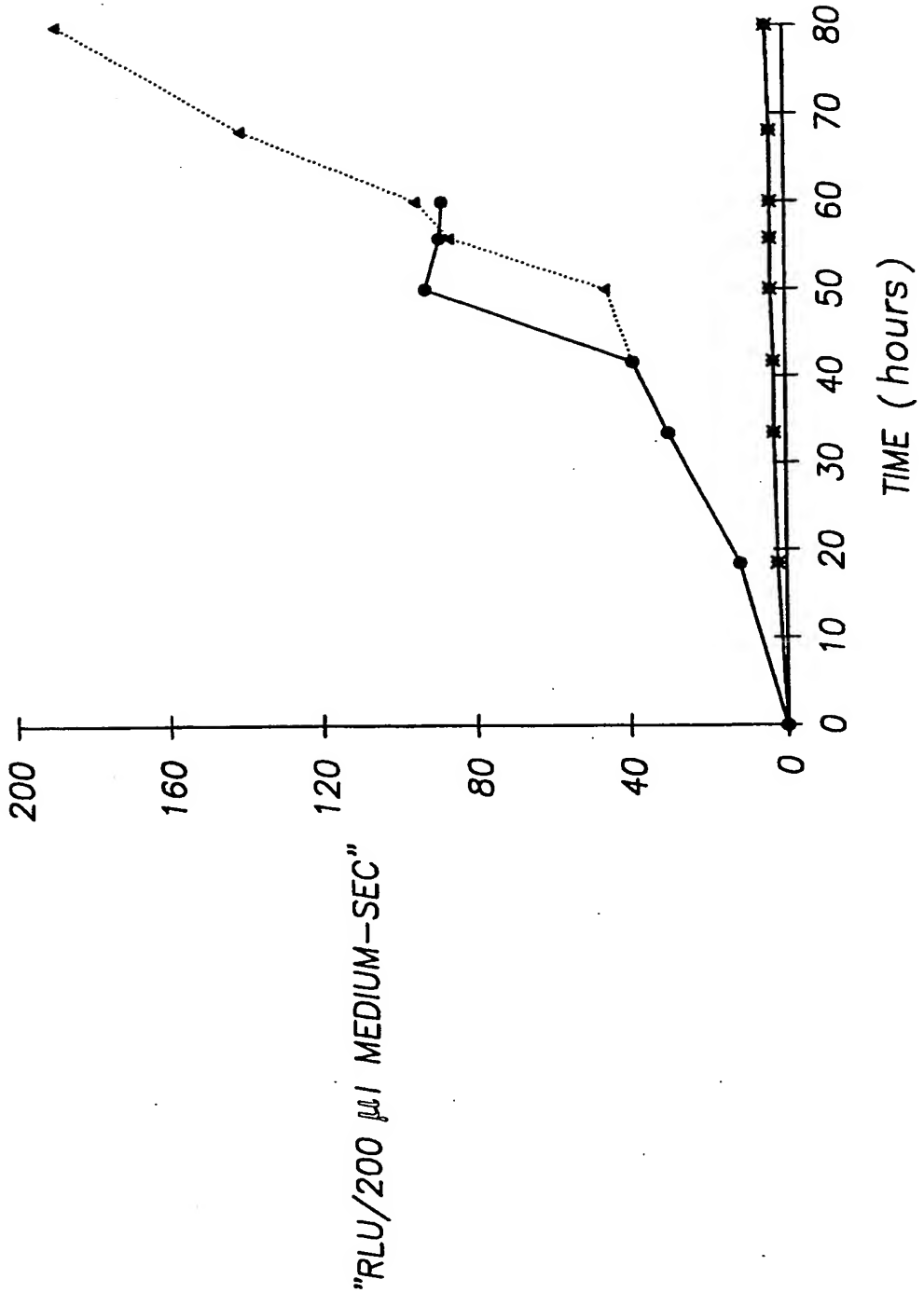
200
160
120
80
40
0
"RLU/200 µl MEDIUM-SEC"
0
10
20
30
40
50
60
70
80
TIME (hours)

FIG.6

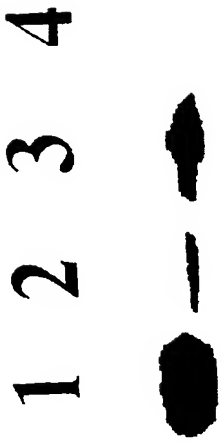


FIG. 7

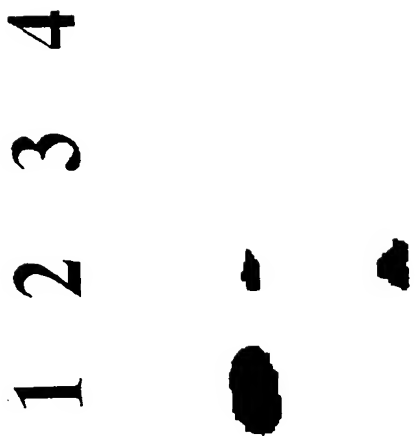


FIG. 8

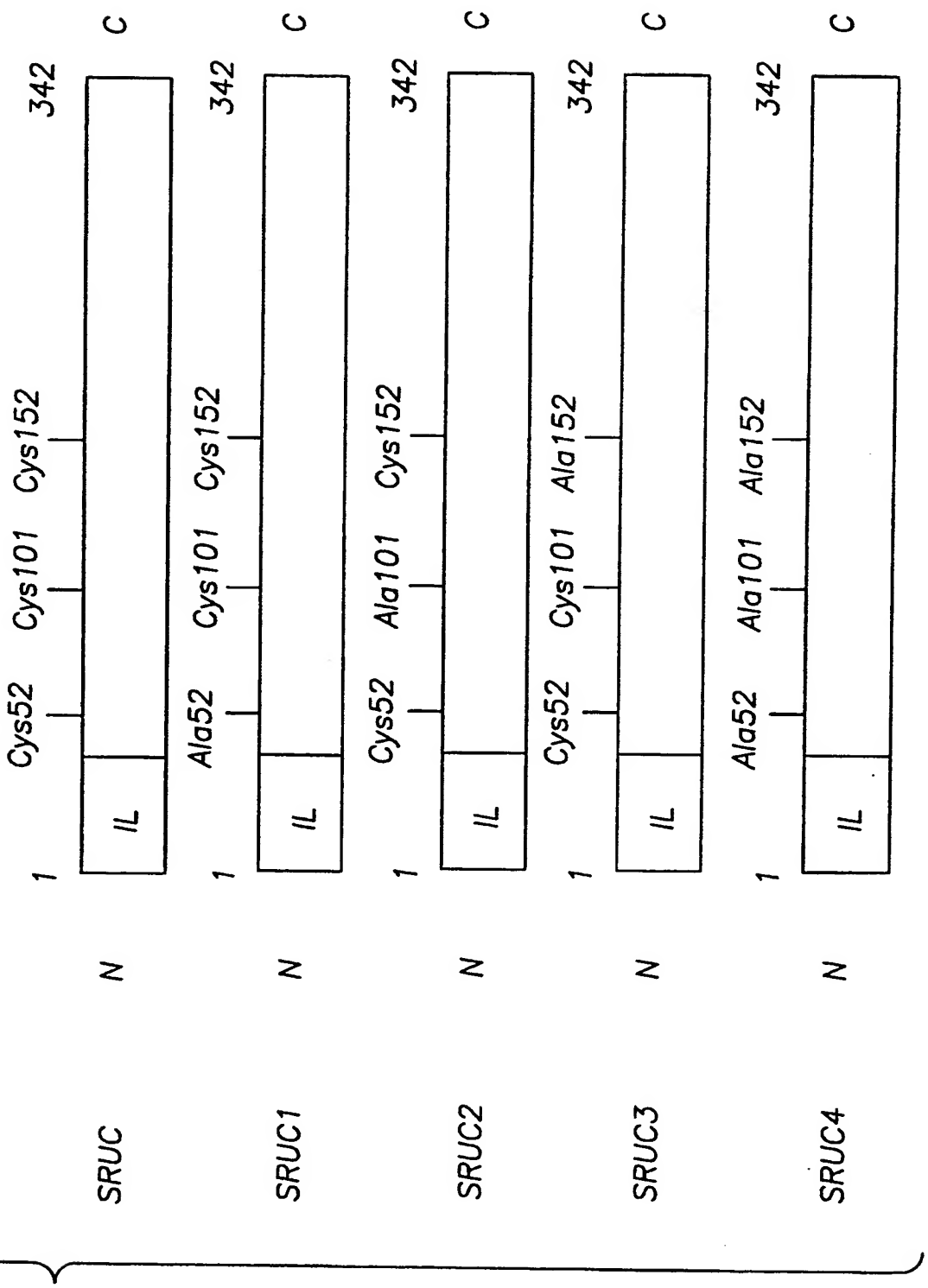


FIG. 9

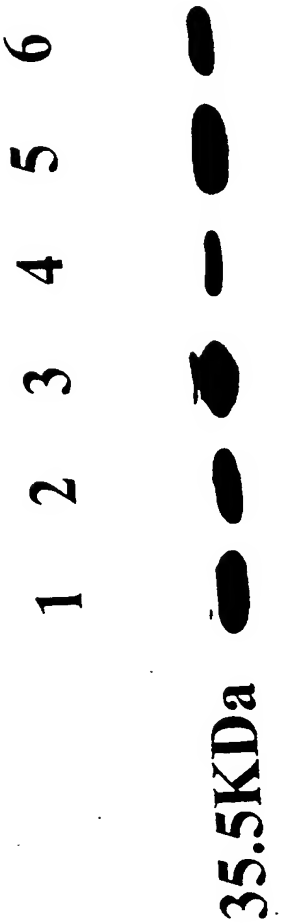


FIG. 10

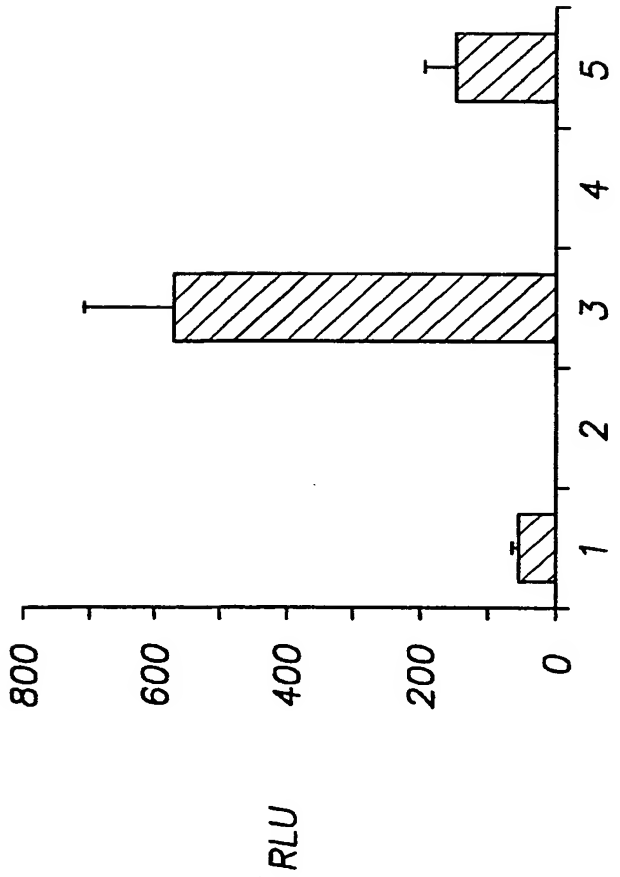


FIG. 11

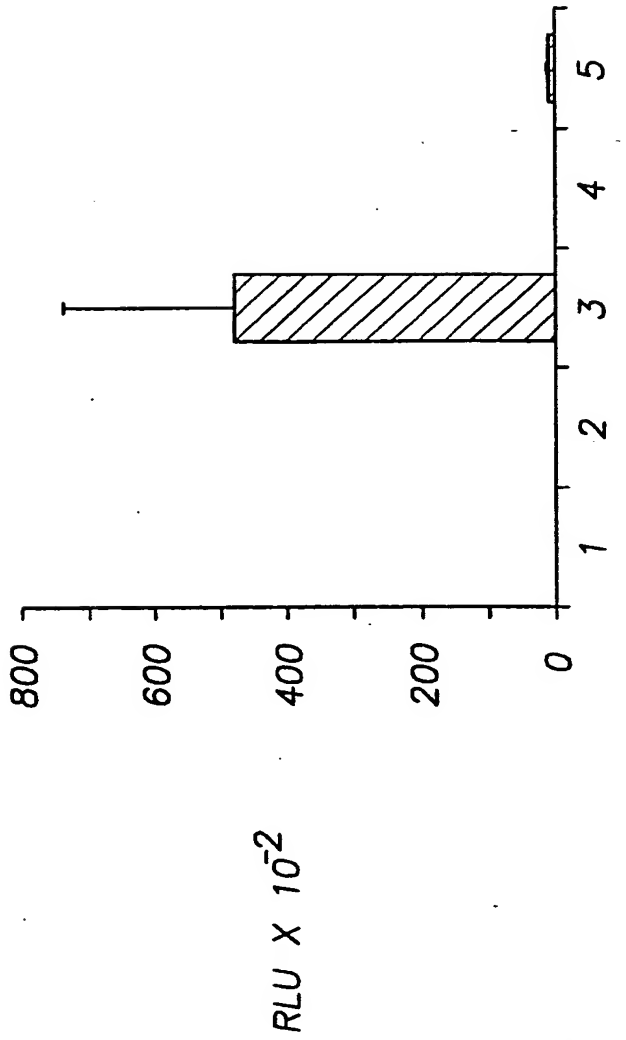


FIG. 12

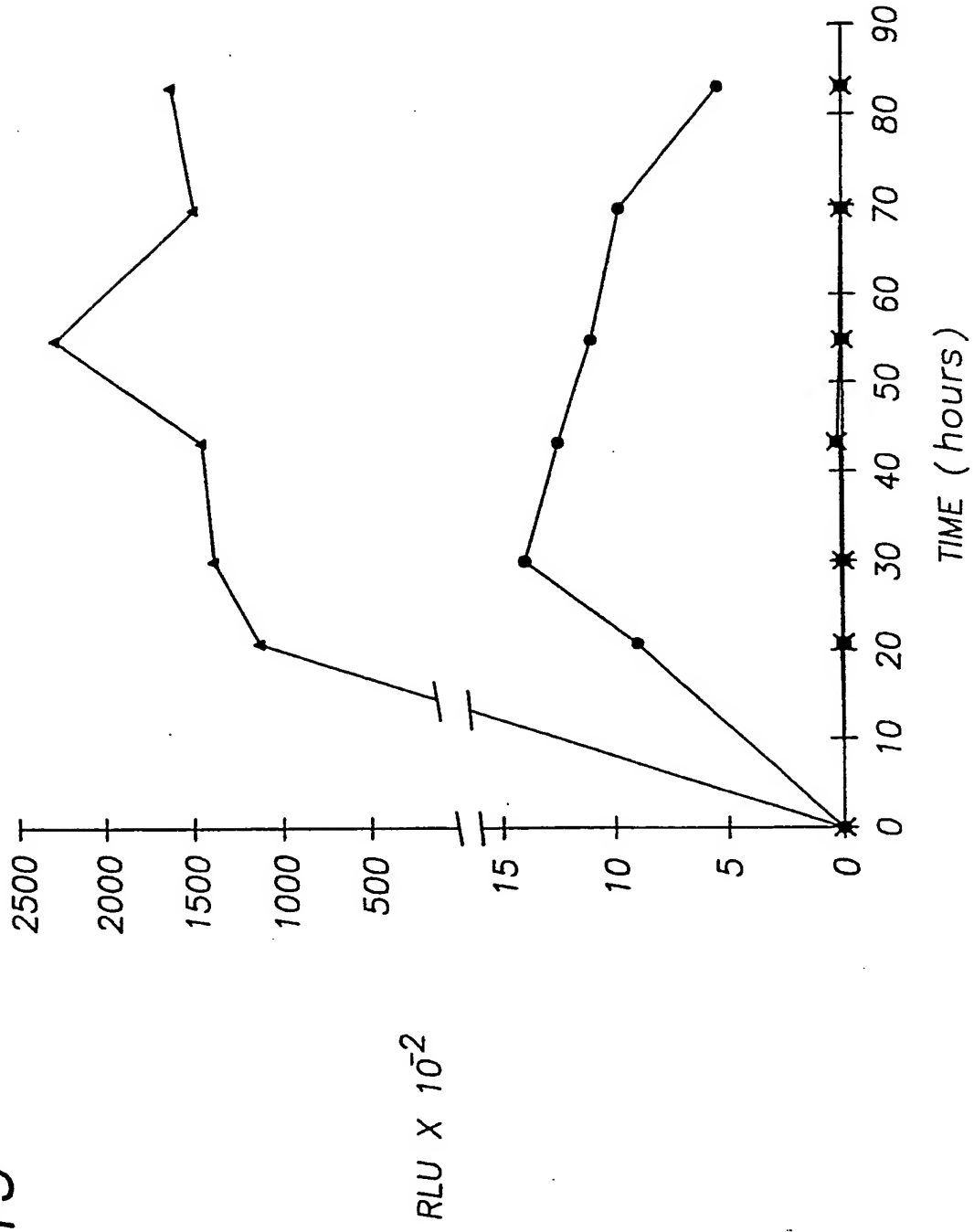


FIG. 13

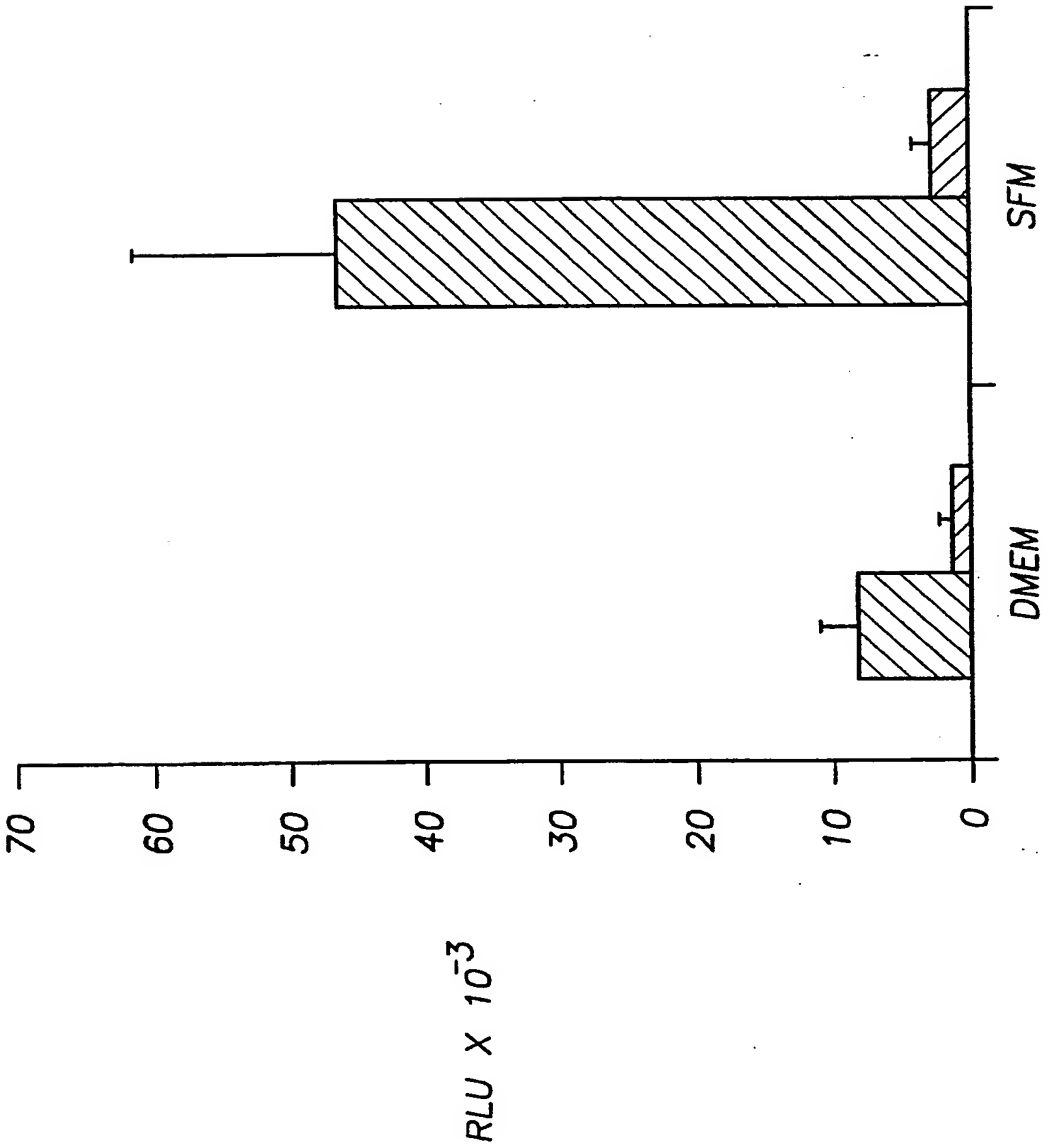


FIG. 14

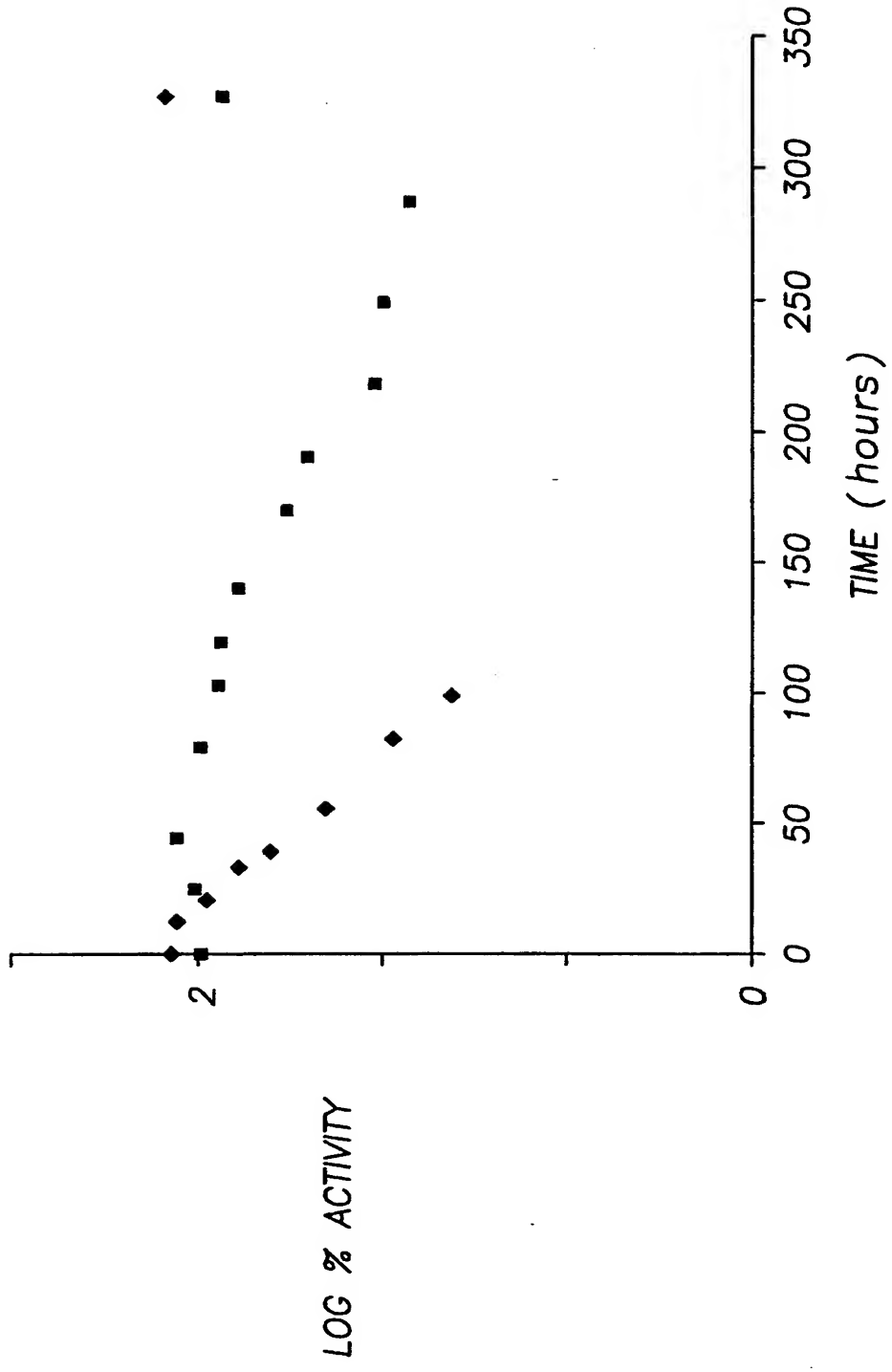


FIG.15

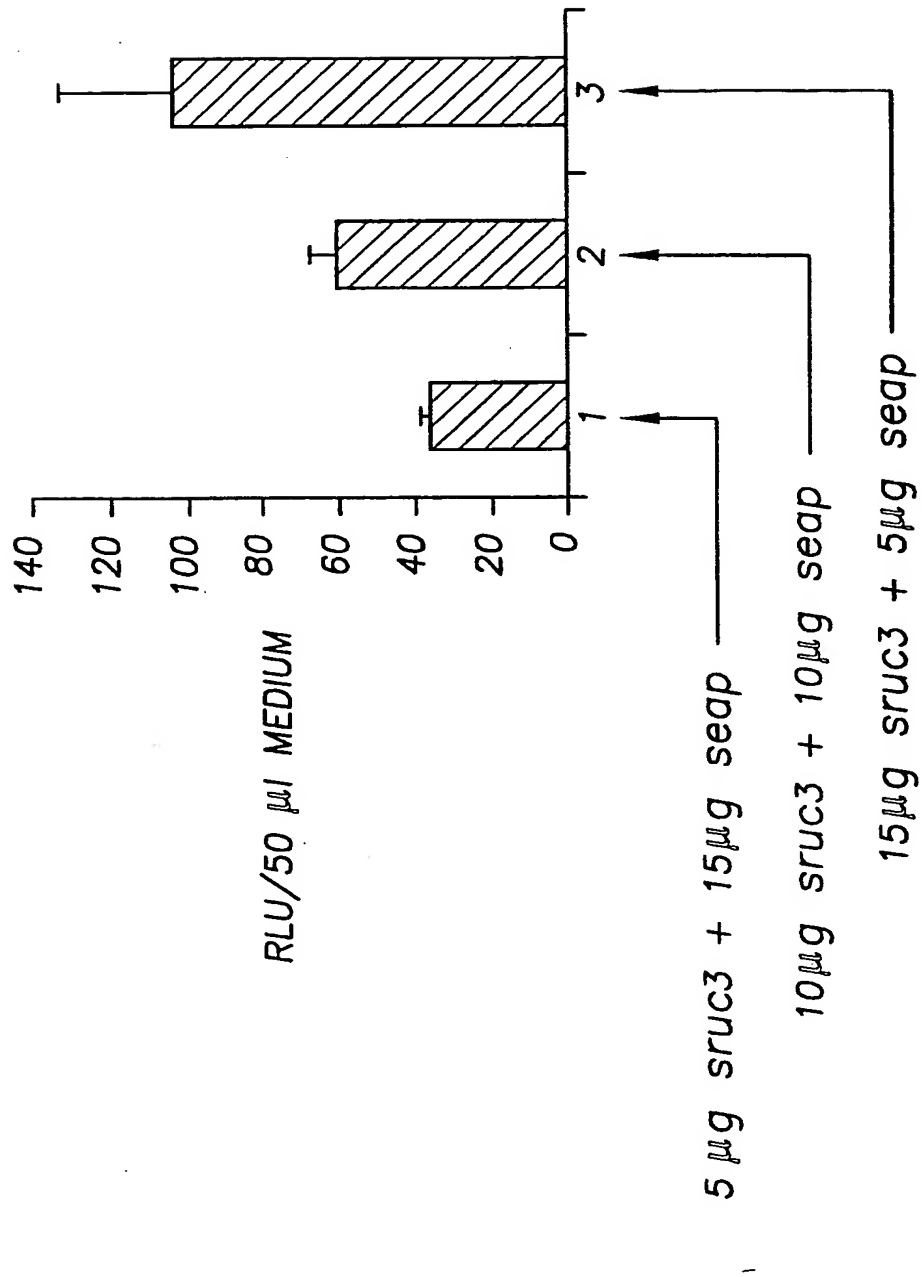
140
120
100
80
60
40
20
0
RLU/50 µl MEDIUM
1
2
3
5 µg sruc3 + 15µg seap
10µg sruc3 + 10µg seap
15µg sruc3 + 5µg seap

FIG. 16

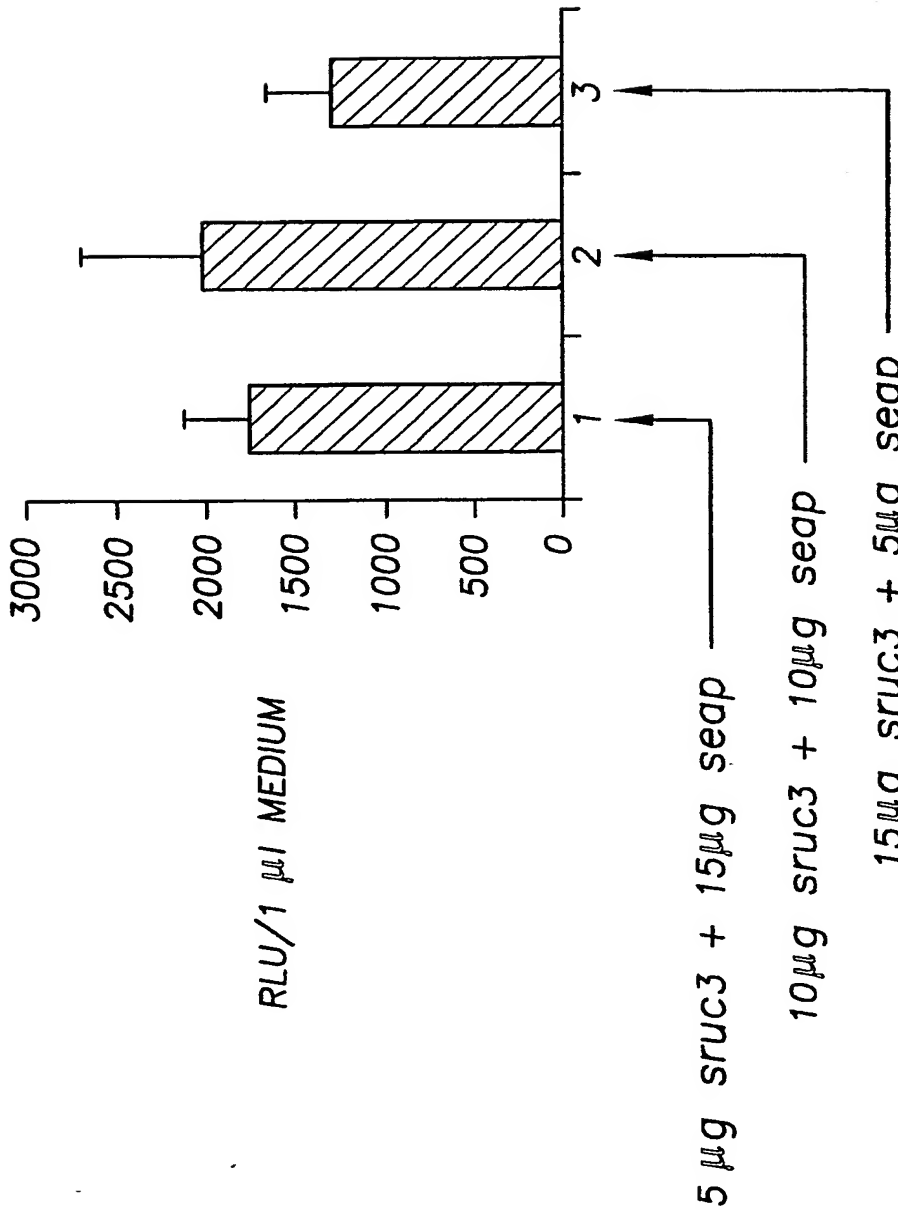
3000
2500
2000
1500
1000
500
0
RLU/1 µl MEDIUM
1
2
3
5 µg sruc3 + 15µg seap
10µg sruc3 + 10µg seap
15µg sruc3 + 5µg seap

FIG. 17

FIG. 18

| PERCENTAGE OF CLONES | LUCIFERASE ACTIVITY |
|---|---|
| 15% | 0–10 RLU |
| 56% | 10–100 RLU |
| 18% | 100–500 RLU |
| 3% | 500–1000 RLU |
| 8% | >1000 RLU |

SEQUENCE LISTING

<110> Escher, Alan P.
Lui, Jingxue
Loma Linda University

<120> Secreted Renilla Liciferase

<130> 12559-2PCT

<140> to be assigned
<141> 1999-09-02

<150> 60/099,214
<151> 1998-09-04

<150> 09/153,031
<151> 1998-09-11

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<151> 1999-06-10

<160> 16

<170> PatentIn Ver. 2.0

<210> 1
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<212> DNA
<213> Renilla reniformis

<220>
<221> CDS
<222> (10)..(945)

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Met Thr Ser Lys Val Tyr Asp Pro Glu Gln Arg Lys Arg Met
1 5 10

ata act ggt ccg cag tgg tgg gcc aga tgt aaa caa atg aat gtt ctt 99
Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val Leu
15 20 25 30

gat tca ttt att aat tat tat gat tca gaa aaa cat gca gaa aat gct 147
Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn Ala
35 40 45

```
gtt att ttt tta cat ggt aac gcg gcc tct tct tat tta tgg cga cat   195
Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg His
                50                  55                  60

gtt gtg cca cat att gag cca gta gcg cgg tgt att ata cca gat ctt   243
Val Val Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp Leu
            65                  70                  75

att ggt atg ggc aaa tca ggc aaa tct ggt aat ggt tct tat agg tta   291
Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu
        80                  85                  90

ctt gat cat tac aaa tat ctt act gca tgg ttt gaa ctt ctt aat tta   339
Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu
    95                  100                 105                 110

cca aag aag atc att ttt gtc ggc cat gat tgg ggt gct tgt ttg gca   387
Pro Lys Lys Ile Ile Phe Val Gly His Asp Trp Gly Ala Cys Leu Ala
                115                 120                 125

ttt cat tat agc tat gag cat caa gat aag atc aaa gca ata gtt cac   435
Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val His
            130                 135                 140

gct gaa agt gta gta gat gtg att gaa tca tgg gat gaa tgg cct gat   483
Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro Asp
        145                 150                 155

att gaa gaa gat att gcg ttg atc aaa tct gaa gaa gga gaa aaa atg   531
Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys Met
    160                 165                 170

gtt ttg gag aat aac ttc ttc gtg gaa acc atg ttg cca tca aaa atc   579
Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys Ile
175                 180                 185                 190

atg aga aag tta gaa cca gaa gaa ttt gca gca tat ctt gaa cca ttc   627
Met Arg Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe
                195                 200                 205

aaa gag aaa ggt gaa gtt cgt cgt cca aca tta tca tgg cct cgt gaa   675
Lys Glu Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu
            210                 215                 220

atc ccg tta gta aaa ggt ggt aaa cct gac gtt gta caa att gtt agg   723
Ile Pro Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val Arg
        225                 230                 235
```

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aat tat aat gct tat cta cgt gca agt gat gat tta cca aaa atg ttt   771
Asn Tyr Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met Phe
    240                 245                 250

att gaa tcg gat cca gga ttc ttt tcc aat gct att gtt gaa ggc gcc   819
Ile Glu Ser Asp Pro Gly Phe Phe Ser Asn Ala Ile Val Glu Gly Ala
255                 260                 265                 270

aag aag ttt cct aat act gaa ttt gtc aaa gta aaa ggt ctt cat ttt   867
Lys Lys Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His Phe
                275                 280                 285

tcg caa gaa gat gca cct gat gaa atg gga aaa tat atc aaa tcg ttc   915
Ser Gln Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe
            290                 295                 300

gtt gag cga gtt ctc aaa aat gaa caa taa ttactttggt tttttattta     965
Val Glu Arg Val Leu Lys Asn Glu Gln
        305                 310

catttttccc gggtttaata atataaatgt cattttcaac aattttattt taactgaata 1025

tttcacaggg aacattcata tatgttgatt aatttagctc gaactttact ctgtcatatc 1085

attttggaat attacctctt tcaatgaaac tttataaaca gtggttcaat taattaatat 1145

atattataat tacatttgtt atgtaataaa ctcggtttta ttataaaaaa a          1196
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<210> 2
<211> 311
<212> PRT
<213> Renilla reniformis

<400> 2

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Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser
             20                  25                  30

Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile
         35                  40                  45

Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val
     50                  55                  60

Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly
```

65 70 75 80

Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp
85 90 95

His Tyr Lys Tyr Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys
100 105 110

Lys Ile Ile Phe Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His
115 120 125

Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu
130 135 140

Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu
145 150 155 160

Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu
165 170 175

Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg
180 185 190

Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu
195 200 205

Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro
210 215 220

Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr
225 230 235 240

Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu
245 250 255

Ser Asp Pro Gly Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys
260 265 270

Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln
275 280 285

Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu
290 295 300

Arg Val Leu Lys Asn Glu Gln
305 310

<210> 3
<211> 1271
<212> DNA
<213> Renilla reniformis

<220>
<221> CDS
<222> (1)..(1017)

<400> 3

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atg tac agg atg caa ctc ctg tct tgc att gca cta agt ctt gca ctt   48
Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
  1               5                  10                  15

gtc aca aac agt gca cct act gaa ttc agc tta aag atg act tcg aaa   96
Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
             20                  25                  30

gtt tat gat cca gaa caa agg aaa cgg atg ata act ggt ccg cag tgg   144
Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
         35                  40                  45

tgg gcc aga tgt aaa caa atg aat gtt ctt gat tca ttt att aat tat   192
Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
     50                  55                  60

tat gat tca gaa aaa cat gca gaa aat gct gtt att ttt tta cat ggt   240
Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
 65                  70                  75                  80

aac gcg gcc tct tct tat tta tgg cga cat gtt gtg cca cat att gag   288
Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
                 85                  90                  95

cca gta gcg cgg tgt att ata cca gat ctt att ggt atg ggc aaa tca   336
Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

ggc aaa tct ggt aat ggt tct tat agg tta ctt gat cat tac aaa tat   384
Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125

ctt act gca tgg ttt gaa ctt ctt aat tta cca aag aag atc att ttt   432
Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
    130                 135                 140

gtc ggc cat gat tgg ggt gct tgt ttg gca ttt cat tat agc tat gag   480
Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
```

```
145                 150                 155                 160

cat caa gat aag atc aaa gca ata gtt cac gct gaa agt gta gta gat   528
His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
                165                 170                 175

gtg att gaa tca tgg gat gaa tgg cct gat att gaa gaa gat att gcg   576
Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190

ttg atc aaa tct gaa gaa gga gaa aaa atg gtt ttg gag aat aac ttc   624
Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
        195                 200                 205

ttc gtg gaa acc atg ttg cca tca aaa atc atg aga aag tta gaa cca   672
Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
    210                 215                 220

gaa gaa ttt gca gca tat ctt gaa cca ttc aaa gag aaa ggt gaa gtt   720
Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

cgt cgt cca aca tta tca tgg cct cgt gaa atc ccg tta gta aaa ggt   768
Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

ggt aaa cct gac gtt gta caa att gtt agg aat tat aat gct tat cta   816
Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

cgt gca agt gat gat tta cca aaa atg ttt att gaa tcg gat cca gga   864
Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
        275                 280                 285

ttc ttt tcc aat gct att gtt gaa ggc gcc aag aag ttt cct aat act   912
Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr
    290                 295                 300

gaa ttt gtc aaa gta aaa ggt ctt cat ttt tcg caa gaa gat gca cct   960
Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro
305                 310                 315                 320

gat gaa atg gga aaa tat atc aaa tcg ttc gtt gag cga gtt ctc aaa   1008
Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
                325                 330                 335

aat gaa caa taattacttt ggtttttttat ttacattttt cccgggttta          1057
Asn Glu Gln
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ataatataaa tgtcattttc aacaatttta ttttaactga atatttcaca gggaacattc 1117

atatatgttg attaatttag ctcgaacttt actctgtcat atcattttgg aatattacct 1177

ctttcaatga aactttataa acagtggttc aattaattaa tatatattat aattacattt 1237

gttatgtaat aaactcggtt ttattataaa aaaa 1271

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<212> PRT
<213> Renilla reniformis

<400> 4

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
1 5 10 15

Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
20 25 30

Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
35 40 45

Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
50 55 60

Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
65 70 75 80

Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
85 90 95

Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
100 105 110

Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
115 120 125

Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
130 135 140

Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
145 150 155 160

His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
165 170 175

```
Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190

Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
        195                 200                 205

Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
    210                 215                 220

Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
        275                 280                 285

Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr
    290                 295                 300

Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro
305                 310                 315                 320

Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
                325                 330                 335

Asn Glu Gln
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<210> 5
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<212> DNA
<213> Artificial Sequence

<220>
<223> DNA fragment used in construction of plasmid

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gcacctactg aattcagctt aaagatg 87

<210> 6
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR Primer

<400> 6
tttgaattca tgtacaggat gcaactcct 29

<210> 7
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 7
tttgaattca gtagtgcact gtttgtgac 29

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 8
tttcccggga aaaatgtaaa taaaaaacca 30

<210> 9
<211> 1271
<212> DNA
<213> Renilla reniformis

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<221> CDS
<222> (1)..(1017)
<223> modified to encode a protein having a cysteine to alanine substitution at position 52

<400> 9

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 1               5                  10                  15

gtc aca aac agt gca cct act gaa ttc agc tta aag atg act tcg aaa   96
Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
            20                  25                  30

gtt tat gat cca gaa caa agg aaa cgg atg ata act ggt ccg cag tgg   144
Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
        35                  40                  45

tgg gcc aga gct aaa caa atg aat gtt ctt gat tca ttt att aat tat   192
Trp Ala Arg Ala Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
    50                  55                  60

tat gat tca gaa aaa cat gca gaa aat gct gtt att ttt tta cat ggt   240
Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
65                  70                  75                  80

aac gcg gcc tct tct tat tta tgg cga cat gtt gtg cca cat att gag   288
Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
                85                  90                  95

cca gta gcg cgg tgt att ata cca gat ctt att ggt atg ggc aaa tca   336
Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

ggc aaa tct ggt aat ggt tct tat agg tta ctt gat cat tac aaa tat   384
Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125

ctt act gca tgg ttt gaa ctt ctt aat tta cca aag aag atc att ttt   432
Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
    130                 135                 140

gtc ggc cat gat tgg ggt gct tgt ttg gca ttt cat tat agc tat gag   480
Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
145                 150                 155                 160

cat caa gat aag atc aaa gca ata gtt cac gct gaa agt gta gta gat   528
His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
                165                 170                 175

gtg att gaa tca tgg gat gaa tgg cct gat att gaa gaa gat att gcg   576
Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190
```

```
ttg atc aaa tct gaa gaa gga gaa aaa atg gtt ttg gag aat aac ttc   624
Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
        195                 200                 205

ttc gtg gaa acc atg ttg cca tca aaa atc atg aga aag tta gaa cca   672
Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
    210                 215                 220

gaa gaa ttt gca gca tat ctt gaa cca ttc aaa gag aaa ggt gaa gtt   720
Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

cgt cgt cca aca tta tca tgg cct cgt gaa atc ccg tta gta aaa ggt   768
Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

ggt aaa cct gac gtt gta caa att gtt agg aat tat aat gct tat cta   816
Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

cgt gca agt gat gat tta cca aaa atg ttt att gaa tcg gat cca gga   864
Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
        275                 280                 285

ttc ttt tcc aat gct att gtt gaa ggc gcc aag aag ttt cct aat act   912
Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr
    290                 295                 300

gaa ttt gtc aaa gta aaa ggt ctt cat ttt tcg caa gaa gat gca cct   960
Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro
305                 310                 315                 320

gat gaa atg gga aaa tat atc aaa tcg ttc gtt gag cga gtt ctc aaa   1008
Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
                325                 330                 335

aat gaa caa taattacttt ggtttttttat ttacattttt cccgggttta          1057
Asn Glu Gln

ataatataaa tgtcattttc aacaatttta ttttaactga atatttcaca gggaacattc 1117

atatatgttg attaatttag ctcgaacttt actctgtcat atcattttgg aatattacct 1177

ctttcaatga aactttataa acagtggttc aattaattaa tatatattat aattacattt 1237

gttatgtaat aaactcggtt ttattataaa aaaa                              1271
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<210> 10
<211> 339
<212> PRT
<213> Renilla reniformis

<400> 10

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 1               5                  10                  15

Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
            20                  25                  30

Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
        35                  40                  45

Trp Ala Arg Ala Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
    50                  55                  60

Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
65                  70                  75                  80

Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
                85                  90                  95

Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125

Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
    130                 135                 140

Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
145                 150                 155                 160

His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
                165                 170                 175

Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190

Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
        195                 200                 205

Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
    210                 215                 220
```

```
Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
        275                 280                 285

Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr
    290                 295                 300

Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro
305                 310                 315                 320

Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
                325                 330                 335

Asn Glu Gln
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<210> 11
<211> 1271
<212> DNA
<213> Artificial Sequence

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<221> CDS
<222> (1)..(1017)
<223> modified to encode a protein having a cysteine to alanine substitution at position 101

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Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
  1               5                  10                  15

gtc aca aac agt gca cct act gaa ttc agc tta aag atg act tcg aaa   96
Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
             20                  25                  30

gtt tat gat cca gaa caa agg aaa cgg atg ata act ggt ccg cag tgg   144
Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
```

```
        35                  40                  45

tgg gcc aga tgt aaa caa atg aat gtt ctt gat tca ttt att aat tat   192
Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
     50                  55                  60

tat gat tca gaa aaa cat gca gaa aat gct gtt att ttt tta cat ggt   240
Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
 65                  70                  75                  80

aac gcg gcc tct tct tat tta tgg cga cat gtt gtg cca cat att gag   288
Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
                 85                  90                  95

cca gta gcg cgg tgt att ata cca gat ctt att ggt atg ggc aaa tca   336
Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

ggc aaa tct ggt aat ggt tct tat agg tta ctt gat cat tac aaa tat   384
Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125

ctt act gca tgg ttt gaa ctt ctt aat tta cca aag aag atc att ttt   432
Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
    130                 135                 140

gtc ggc cat gat tgg ggt gct gct ttg gca ttt cat tat agc tat gag   480
Val Gly His Asp Trp Gly Ala Ala Leu Ala Phe His Tyr Ser Tyr Glu
145                 150                 155                 160

cat caa gat aag atc aaa gca ata gtt cac gct gaa agt gta gta gat   528
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ttc gtg gaa acc atg ttg cca tca aaa atc atg aga aag tta gaa cca   672
Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
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ggt aaa cct gac gtt gta caa att gtt agg aat tat aat gct tat cta   816
Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

cgt gca agt gat gat tta cca aaa atg ttt att gaa tcg gat cca gga   864
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305                 310                 315                 320

gat gaa atg gga aaa tat atc aaa tcg ttc gtt gag cga gtt ctc aaa  1008
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Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
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Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
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Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
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Val Gly His Asp Trp Gly Ala Ala Leu Ala Phe His Tyr Ser Tyr Glu
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His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
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Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
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Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
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Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
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Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
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Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
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Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
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<213> Renilla reniformis

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<221> CDS
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tgg gcc aga tgt aaa caa atg aat gtt ctt gat tca ttt att aat tat   192
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Pro Val Ala Arg Ala Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

ggc aaa tct ggt aat ggt tct tat agg tta ctt gat cat tac aaa tat   384
Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125

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    130                 135                 140

gtc ggc cat gat tgg ggt gct tgt ttg gca ttt cat tat agc tat gag   480
Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
145                 150                 155                 160

cat caa gat aag atc aaa gca ata gtt cac gct gaa agt gta gta gat   528
His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
                165                 170                 175

gtg att gaa tca tgg gat gaa tgg cct gat att gaa gaa gat att gcg   576
Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190

ttg atc aaa tct gaa gaa gga gaa aaa atg gtt ttg gag aat aac ttc   624
Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
        195                 200                 205

ttc gtg gaa acc atg ttg cca tca aaa atc atg aga aag tta gaa cca   672
Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
    210                 215                 220

gaa gaa ttt gca gca tat ctt gaa cca ttc aaa gag aaa ggt gaa gtt   720
Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

cgt cgt cca aca tta tca tgg cct cgt gaa atc ccg tta gta aaa ggt   768
Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

ggt aaa cct gac gtt gta caa att gtt agg aat tat aat gct tat cta   816
Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

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Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro
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gat gaa atg gga aaa tat atc aaa tcg ttc gtt gag cga gtt ctc aaa  1008
Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
                325                 330                 335

aat gaa caa taattacttt ggttttttat ttacattttt cccgggttta          1057
Asn Glu Gln

ataatataaa tgtcattttc aacaatttta ttttaactga atatttcaca gggaacattc 1117

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         35                  40                  45

Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
     50                  55                  60

Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
 65                  70                  75                  80
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Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
85 90 95

Pro Val Ala Arg Ala Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
100 105 110

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Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu
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His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
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Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
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195 200 205

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Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
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<223> modified to encode a protein having a cysteine to alanine substitution at positions 52, 101 and 152

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Val Thr Asn Ser Ala Pro Thr Glu Phe Ser Leu Lys Met Thr Ser Lys
             20                  25                  30

gtt tat gat cca gaa caa agg aaa cgg atg ata act ggt ccg cag tgg   144
Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp
         35                  40                  45

tgg gcc aga tgt aaa caa atg aat gtt ctt gat tca ttt att aat tat   192
Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr
     50                  55                  60

tat gat tca gaa aaa cat gca gaa aat gct gtt att ttt tta cat ggt   240
Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
 65                  70                  75                  80

aac gcg gcc tct tct tat tta tgg cga cat gtt gtg cca cat att gag   288
Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
                 85                  90                  95

cca gta gcg cgg tgt att ata cca gat ctt att ggt atg ggc aaa tca   336
Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
            100                 105                 110

ggc aaa tct ggt aat ggt tct tat agg tta ctt gat cat tac aaa tat   384
Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
        115                 120                 125
```

```
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Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
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gtc ggc cat gat tgg ggt gct gct ttg gca ttt cat tat agc tat gag   480
Val Gly His Asp Trp Gly Ala Ala Leu Ala Phe His Tyr Ser Tyr Glu
145                 150                 155                 160

cat caa gat aag atc aaa gca ata gtt cac gct gaa agt gta gta gat   528
His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu Ser Val Val Asp
                165                 170                 175

gtg att gaa tca tgg gat gaa tgg cct gat att gaa gaa gat att gcg   576
Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala
            180                 185                 190

ttg atc aaa tct gaa gaa gga gaa aaa atg gtt ttg gag aat aac ttc   624
Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe
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ttc gtg gaa acc atg ttg cca tca aaa atc atg aga aag tta gaa cca   672
Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro
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gaa gaa ttt gca gca tat ctt gaa cca ttc aaa gag aaa ggt gaa gtt   720
Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val
225                 230                 235                 240

cgt cgt cca aca tta tca tgg cct cgt gaa atc ccg tta gta aaa ggt   768
Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly
                245                 250                 255

ggt aaa cct gac gtt gta caa att gtt agg aat tat aat gct tat cta   816
Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu
            260                 265                 270

cgt gca agt gat gat tta cca aaa atg ttt att gaa tcg gat cca gga   864
Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly
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Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile Phe Leu His Gly
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Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val Pro His Ile Glu
85 90 95

Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser
100 105 110

Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr
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Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe
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305 310 315 320

Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys
325 330 335

Asn Glu Gln

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, C12N 1/21, 5/06, 9/02, 15/52, C07H 21/04 — A3

(11) International Publication Number: **WO 00/20619**

(43) International Publication Date: 13 April 2000 (13.04.00)

(21) International Application Number: PCT/US99/20093

(22) International Filing Date: 2 September 1999 (02.09.99)

(30) Priority Data:

| | | |
|---|---|---|
| 09/099,214 | 4 September 1998 (04.09.98) | US |
| 09/330,317 | 10 June 1999 (10.06.99) | ** |

(71) Applicant *(for all designated States except US)*: LOMA LINDA UNIVERSITY [US/US]; Loma Linda, CA 92350 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: ESCHER, Alan, P. [FR/US]; 463 Jefferson Street, Redlands, CA 92374 (US). LIU, Jingxue [CN/US]; 25158 Crest View Drive, Loma Linda, CA 92354 (US).

(74) Agents: SHELDON, Jeffrey, G. et al.; Sheldon & Mak, Inc., 9th floor, 225 South Lake Avenue, Pasadena, CA 91101 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(88) Date of publication of the international search report:
6 July 2000 (06.07.00)

(54) Title: SECRETED *RENILLA* LUCIFERASE

(57) Abstract

A polynucleotide encoding a secreted form of wild type *Renilla* luciferase. Also provided is a polynucleotide encoding a secreted modified form of wild type *Renilla* luciferase. Additionally, the polypeptides encoded by the polynucleotides of the present invention and uses of the polynucleotides and polypeptides of the present invention in biological assays. Also, a stable mammalian packaging cell line which produces retroviruses carrying a polynucleotide encoding a secreted *Renilla* luciferase.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakstan | RO | Romania | | |
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| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12N 1/21, 5/06, 9/02, 15/52; C07H 21/04

US CL :435/6, 189, 252.3, 320.1, 325, 810; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 189, 252.3, 320.1, 325, 810; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN (CAPLUS, BIOSIS, BIOTECHDS, NITS, SCISEARCH)
search terms: luciferase, secret?, Renilla

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y<br>---<br>A | LIU et al. Secretion of Functional Renilla reniformis Luciferase by mammalian cells. Gene. 12 December 1997, Vol. 203, pages 141-148, the entire document. | 1-4, 6-9, 11, 12, 14-17, 22, 25, 28-32, 35, 38<br>-------<br>19-21, 23, 26, 27, 33, 34, 36, 37<br>----------<br>5, 10, 13, 18, 39 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>03 APRIL 2000 | Date of mailing of the international search report<br>01 MAY 2000 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>ELIZABETH SLOBODYANSKY<br>Telephone No. (703) 308-0196<br>JOYCE BRIDGERS<br>PARALEGAL SPECIALIST<br>CHEMICAL ISATRIX |

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20093

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y<br>----<br>A | LORENZ et al. Isolation and Expression of a cDNA encoding Renilla reniformis Luciferase. Proc. Natl. Acad. Sci. USA. May 1991, Vol. 88, pages 4438-4442, especially pages 4438-4440. | 1-4, 6-9, 11, 12, 14-17, 19-38<br>-----<br>5, 10, 13, 18, 39 |
| Y<br>----<br>A | INOUYE et al. Imaging of Luciferase Secretion from Transformed Chinese Hamster Ovary Cells. Proc. Natl. Acad. Sci. USA. October 1992, Vol. 89, pages 9584-9587, especially abstract and page 9584, 2nd column, 1st paragraph; page 9587, 1st column, 2nd paragraph. | 1-4, 6-9, 11, 12, 14-17, 19-38<br>---------<br>5, 10, 13, 18, 39 |
| Y<br>----<br>A | THOMPSON et al. Vargula hilgendorfii Luciferase: a Secreted Reporter Enzyme for Monitoring Gene expression in Mammalian Cells. Gene. 1990, Vol. 96, pages 257-262, especially abstract and page 258, 2nd column, 2nd paragraph. | 1-4, 6-9, 11, 12, 14-17, 19-38<br>---------<br>5, 10, 13, 18, 39 |
| Y<br>---<br>A | LU et al. Secretion of Firefly Luciferase in E. coli. Biotechnology Letters. November 1993, Vol. 15, No. 11, pages 1111-1116, especially page 1111, last paragraph and pages 1113-1115. | 1-4, 6-9, 11, 12, 14-17, 19-38<br>------<br>5, 10, 13, 18, 39 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20093

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20093

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-5,11-24,28-39, drawn to a polynucleotide encoding a secreted Renilla luciferase, a vector containing it, a cell transformed with the same and a method of use thereof.
Group II, claim(s) 6-10 and 25-27, drawn to a polypeptide encoded by said polynucleotide.
The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: a DNA of Group I and a polypeptide of Group II each considered as a whole do not make a contribution over the prior art as evidenced by Liu et al.

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WO 00/20619 A3

(54) Title: SECRETED *RENILLA* LUCIFERASE

(57) Abstract: A polynucleotide encoding a secreted form of wild type *Renilla* luciferase. Also provided is a polynucleotide encoding a secreted modified form of wild type *Renilla* luciferase. Additionally, the polypeptides encoded by the polynucleotides of the present invention and uses of the polynucleotides and polypeptides of the present invention in biological assays. Also, a stable mammalian packaging cell line which produces retroviruses carrying a polynucleotide encoding a secreted *Renilla* luciferase.